STUDIES OF THE DIAGNOSIS AND IMMUNOPATHOGENESIS OF WEGENER'S GRANULOMATOSIS

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ABSTRACT

Wegener's Granulomatosis, classically, comprises a triad of granulomatous vasculitis in the upper and lower respiratory tracts, and a focal and segmental, necrotising glomerulonephritis in the kidney. In practice disease presentation and organ involvement is widespread and variable. The aetiology is unknown but an infectious aetiology has been proposed, based on the especial involvement of the respiratory tract in the disease process. The pathogenesis is also unclear but immune complex deposition leading to neutrophil chemotaxis and activation causing endothelial injury has been suggested. Recently antibodies against a component of neutrophil cytoplasm have been described in Wegener's Granulomatosis. This thesis records studies of the diagnosis and pathogenesis of Wegener's Granulomatosis. The first part of the study examined the problem of diagnosis using renal biopsy material. Renal biopsy is important because renal functional status is the major factor determining outcome, yet renal biopsy appearances are not specific for the condition and may be found in other vasculitides such as microscopic polyarteritis. Review of the histology, immunofluorescence studies and ultrastructure of renal biopsies from patients with Wegener's Granulomatosis and microscopic polyarteritis revealed no diagnostically useful differences. In Wegener's Granulomatosis renal mast cells

were frequently present unassociated with areas of active inflammation, whereas in microscopic polyarteritis they were predominantly present as part of an inflammatory infiltrate. In both conditions the number of mast cells was increased. The functional significance of this difference is unclear.

The second part of the study examined the presence of autoantibodies against neutrophils. IgG antibodies giving coarse, granular, cytoplasmic fluorescence when incubated with cytospin preparations of normal neutrophils were found to be highly specific for Wegener's Granulomatosis. Diffuse cytoplasmic fluorescence was present in a wide variety of other diseases including some other forms of systemic vasculitis. By differential protein extraction of neutrophils and Western Blot analysis IgG antibodies which gave coarse fluorescence were found to react with 45 kDa and 27-31 kDa proteins in the membrane-bound protein extract. This is consistent with the autoantibodies being directed against a component of neutrophil granules.

An hypothesis is proposed. Wegener's Granulomatosis is the product of an immunological response to an antigen, possibly to an inhaled, exogenous pathogen. A predominantly cell-mediated response results in the typical lesions identified pathologically within the respiratory tract including granulomata. A humoral response also may

occur, reflected by the presence of specific autoantibodies and this can lead to systemic injury primarily by the formation and deposition of immune complexes. In other systemic vasculitides, such as microscopic polyarteritis, a variety of exogenous antigens result in humoral responses and immune complex formation and deposition leading to the same pattern of renal injury.

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STATEMENT OF ORIGINALITY

The work recorded in this thesis is my own, and was performed by myself or under my direct supervision.

Dr. Mary MacDonald and Miss Claire Neary, a BSc Honours student, were involved in some aspects of the studies of renal biopsies including morphology, ultrastructure and mast cell distribution.

Mr. Robert Simpson helped to develop the immunofluorescence assay for antibodies to neutrophil cytoplasmic antigens, and also helped with the ELISA test. Mr. Rajesh Kharbanda, a BSc Honours student, contributed to the immunoblotting experiments.

CONTENTS

CHAPTER 1

INTRODUCTION

DESCRIPTION OF WEGENER'S GRANULOMATOSIS

Wegener's Granulomatosis (WG) is a syndrome classically characterised by a triad of necrotising granulomatous vasculitis of the upper and lower respiratory tracts and a focal and segmental necrotising glomerulonephritis. There may also be disseminated small vessel vasculitis (Wegener, 1931; Wegener, 1938; Godman and Churg, 1958; Crofton and Douglas, 1982; Brennar and Lezarrus, 1988).

Clinical Features

The disease is reviewed in a number of publications (Walton, 1958; Matthay, 1980; Appel et al, 1981; Fauci et al, 1977; Fauci et al, 1983; Hall, 1985)

The disease can occur at any age, but is more common in adults: the average age at onset of symptoms is about 40 years and males are more commonly affected than females.

The clinical manifestation of WG is related to the anatomical location of vessels involved and the degree of vasculitis. Since potentially any small vessel in the body can be affected, symptoms may arise from any organ system. There is thus a spectrum of clinical findings associated with WG (De Remee et al, 1986; Cupps and Fauci, 1981; Fauci et al, 1983; Pinching et al, 1983).

Onset of disease is usually insidious and accompanied by

constitutional symptoms such as fever, weight loss, malaise and fatigue (Fauci et al, 1973; Howell and Epstein, 1976; Van der Woude et al, 1982)

The upper respiratory tract is commonly affected early in disease giving rise to symptoms such as rhinorrhoea, nasal congestion, epistaxis, crusting, purulent discharge and sinus pain. Such symptoms may be attributed to allergic rhinitis and sinusitis - thereby delaying the true diagnosis. Involvement of the upper respiratory tract is present in about 60% of cases at presentation (Parlevliet et al, 1988). Ulceration of the mucosa and secondary infection, orbital infiltration and destruction of the nasal cartilage to result in a "saddle nose" deformity are all possible complications.

Hearing loss, which may be the presenting complaint, occurs in up to 60% of untreated cases from Eustachian tube obstruction and otitis media, but rarely due to granulomatous infiltration of the middle ear. Ocular complications include conjunctivitis and scleritis.

Involvement of the lower respiratory tract is usual as the disease progresses and is characterised by symptoms such as chronic productive cough, haemoptysis, dyspnoea and pleuritic pain. The pulmonary system is affected in most patients - 94% developing functional or radiographic evidence of lung disease (Matthay, 1980; Leavitt and

Fauci, 1986). Pulmonary radiographic abnormalities are variable: they are typically nodular, but may be diffuse and can involve any number of lobes and may be unilateral or bilateral. Massive pulmonary infiltration with respiratory failure and life threatening lung haemorrhage are the major complications associated with disease of the respiratory system (Hensley, 1977).

Skin lesions are common clinical manifestation of WG (Ronco et al, 1983). Most lesions are ulcerative but they may be papular or vesicular and subcutaneous nodules and palpable purpura may also occur (Copeman et al, 1970).

Other, less common clinical manifestations of Wegener's Granulomatosis are pericarditis, endocarditis, coronary vasculitis, cerebral vasculitis and even diabetes insipidus.

Renal involvement in Wegener's Granulomatosis is usually associated with widespread dissemination of the disease and may occur late. The degree of renal disease at presentation is a major factor determining outcome. At presentation many patients are found to show evidence of glomerular injury in the form of microscopic haematuria, but the time over which significant renal disease develops is extremely variable (Serra et al, 1984). Extrarenal symptoms often precede clinical renal disease, but cases where renal symptoms are the only manifestation of disease

(Van der Woude et al, 1982), reflect the spectrum of the clinical syndrome of WG.

Pathology

Lesions in the upper and lower respiratory tract take the form of focal necrotising granulomatous lesions which may become confluent. Such lesions occur in the nasal and oral cavities, paranasal sinuses, larynx, trachea and also scattered throughout the lung parenchyma (Robbins et al, 1984).

The focal areas of necrosis are generally surrounded by a zone of fibroblastic proliferation with giant cell and mixed inflammatory cell infiltration. These lesions undergo progressive fibrosis and organisation (Godman and Churg, 1958; Isreal et al, 1977; Robbins et al, 1984). The vasculitis is predominantly fibrinoid necrosis of small arteries and veins with early infiltration by neutrophils followed by mononuclear cells, and healing with fibrosis (Godman and Churg, 1958). Whilst common sites to be affected are the respiratory tract and kidney, vasculitis may occur in spleen, skin, liver, epididymis, prostate and lymph nodes (Godman and Churg, 1958; Walton, 1958).

The classical renal appearances of a focal and segmental necrotising glomerulonephritis may not be identified in all renal biopsies, but there is often a degree of diffuse mesangial proliferation (Robbins et al, 1984). Crescentic

glomerulonephritis may be superimposed on the necrotising lesion (Heptinstall, 1983), and in older lesions segmental or total glomerulosclerosis may occur. Necrotising vasculitis is rarely demonstrated in biopsy material but may be demonstrated at autopsy (Heptinstall, 1983; Brennar and Lezarrus, 1988). Granulomata are rarely demonstrated (Godman and Churg, 1958; Appel et al, 1981; Novak, Christiansen and Sorenson, 1982; Weiss et al, 1984).

Immunofluorescence studies on renal biopsies are not in agreement. In some cases focal deposits of IgG and C3 have been identified (Baker et al, 1978), as have deposits of C3 alone (Ronco et al, 1983). However, many studies have not found immune deposits (McIntosh, 1971; Juncos et al, 1979; McClusky and Feinburg, 1983; Brennar and Lezarrus, 1988). Fibrinogen can be demonstrated in areas of necrosis and in crescents (Howell and Epstein, 1976; Hensley, 1979; Juncos et al, 1979; Ronco et al, 1984). The origin of glomerular crescents is not clear.

Electron microscopy studies demonstrated focal disruption of the glomerular basement membrane and small subendothelial deposits (Aldo and Benson, 1970; Juncos et al, 1979).

Treatment and Course

Untreated WG is a rapidly fatal condition (Kjellstrand et

al, 1974): the mean survival time of patients with generalised untreated WG is five months, with over 80% mortality in 1 year and over 90% in two years (Walton, 1958; Aldo and Benson, 1970). However, the time course over which there is progression from the "limited" form of the disease - defined as consisting of only pulmonary involvement - to the generalised disease is extremely variable (De Remee et al, 1976; Appel et al, 1981; Feinberg, 1981; Pinching et al, 1983). Around 85% of patients treated survive for 1 year (Coward et al, 1986).

The use of corticosteroid therapy alters the survival time to a mean of 12.5 months with a 1 year mortality of 66% (Aldo and Benson, 1970). However, immunosuppressive therapy has improved the prognosis markedly (Reza et al, 1976; Fauci et al, 1983). Chronic low dose cyclophosphamide therapy (2 mg/kg body weight) induces remission in 93% of cases with an average duration of 4 years (Fauci et al, 1983). A proportion of these patients subsequently remain in remission for around three years without therapy (Fauci et al, 1983).

Immunosuppressive therapy is toxic and side effects are serious. It increases the risk from opportunistic infections, which may be fulminant and fatal (Fauci, 1983). gonadal dysfunction, haemorrhagic cystitis and hair loss are all common complications (Fauci et al, 1983. There have also been reports of leukaemia (Wheeler, 1981;

Ohyashiki et al, 1986) and lymphoma (Sart et al; 1983; Ambrus et al, 1984; Colburn et al, 1985) in WG patients following treatment with immunosuppressive therapy. Such therapy should not be administered inappropriately and this emphasises the need for correct diagnosis.

In addition immunosuppressive therapy does not induce remission in all cases, especially where there is advanced renal disease or presentation with acute renal failure (Kjellstrand et al, 1974; Appel et al, 1981; Pinching et al, 1983). Mortality rates of 44% at 3 years (Pinching et al, 1983) and 50% at 5 years (Appel et al, 1981) have been reported for patients on treatment.

Early disease with severe acute injury appears more responsive to therapy (Wolff et al, 1974), indicating the necessity for early diagnosis.

Diagnosis

Diagnosis of WG is based upon a combination of typical clinical findings and characteristic histology (Howell and Epsten, 1976; Appel et al, 1981; Fauci et al, 1983; Pinching et al, 1983; Serra et al, 1984; Parlevliet, 1988). In view of the spectrum of clinical features associated with WG, diagnosis cannot be established on clinical findings alone. Diagnostic histological evidence of WG is the presence of granulomatous lesions in the upper

and lower respiratory tract. Renal involvement is not always evident or specific. Lesions are focal so that the biopsied area may not contain diagnostic tissue; the diversity of clinical presentation means that certain biopsied tissue may not be involved in the disease process. If only the kidney is involved the diagnosis of WG cannot be established since the histological findings are identical to those of microscopic polyarteritis (Heptinstall, 1983).

Aetiology and Pathogenesis

The aetiology of WG is not known and the pathogenesis is unclear. An immunological mechanism is thought to be involved in the disease process (Fauci et al, 1983) and there is an increased frequency of subjects with HLA-B8 (Katz et al, 1979) and HLA-DR2 (Elkon et al, 1983). However, the literature is scanty and speculative and no comprehensive picture exists.

A type IV delayed hypersensitivity type reaction has been proposed to account for the lesions in the respiratory tract (Berman et al, 1963; Fauci et al, 1971; Douglas et al, 1976) and a type III hypersensitivity reaction to explain the systemic vasculitis (Fauci, 1978). However, immune complexes are not demonstrated in all lesions (Isreal et al, 1977; Ronco et al, 1983) and their significance in pathogenicity remains unclear (McClusky and

Feinburg, 1983).

A role for polymorphonuclear leucocytes is suggested by models of immune complex disease in experimental animals (Henson and Cochrane, 1971). The granule contents of neutrophils are potentially able to cause vascular injury (Harlan et al, 1981) and in particular elastase is able to detach and lyse endothelial cells in vitro (Harlan et al, 1981; Smedley et al, 1986) and degrade subendothelial matrix even in the presence of alpha-1-proteinase inhibitor (Weiss and Regiani, 1984). Ultrastructurally intravascular lysis of neutrophils has been observed in active disease (Donald et al, 1976).

Recently the discovery of IgG autoantibodies directed against a component of neutrophil cytoplasm (Van der Woude et al, 1985) has led to hopes of more accurate diagnosis of WG and a fuller understanding of pathogenesis.

Aims

This thesis is the result of studies of WG with particular reference to diagnosis of the disease, and an attempt to elucidate immunopathogenetic mechanisms involved.

Chapter 2 centres on the nature and significance of renal changes in WG, in terms of both diagnosis and possible pathogenesis.

Chapter 3 of the thesis examines the recently described autoantibodies in WG; in diagnosis, monitoring of disease activity and nature of the target antigens.

Chapter 4 attempts to interpret these new data and speculates on the nature of immune responses implicated in WG.

CHAPTER 2

RENAL BIOPSY IN WEGENER'S GRANULOMATOSIS

2.1.1 INTRODUCTION

The first description of WG in 1931 by Klinger described it as a variant of polyarteritis. Since then the relationship between these two conditions has continued to be controversial and unclear (Berman et al, 1963; Savage et al, 1985; Rasmussen et al, 1987). Both conditions are systemic necrotising vasculitides (Davidson et al, 1948) with a poor prognosis if not treated rapidly (Walton, 1958). Whereas granulomata are present in respiratory tract lesions in WG they are not present in MPA . This has led to the suggestion that Type IV delayed hypersensitivity is involved in the pathogenesis of WG (Berman et al, 1963; Fauci et al, 1971; Douglas et al, 1976). MPA is thought to be caused by immune complex deposition as part of Type III hypersensitivity reaction (Magil et al, 1978; Freedman et al, 1960; Rich, 1942, (a); Rich, 1942 (b); Gocke et al, 1970) although the aetiological antigen is frequently unknown (Brentjens et al, 1979). Most reports have found no difference between the renal biopsy findings in WG and MPA (Dunnill, 1984; Heptinstall, 1983; Cupps and Fauci, 1981). In particular most authors have not found granulomata in WG (Novak et al, 1984) although some have (Pinching et al, 1983). Because different hypersensitivity reactions may be involved in the pathogenesis of WG and MPA renal biopsy material from patients WG and MPA was reviewed by light microscopy,

immunofluorescence and electron microscopy.The findings were compared to discover (i) whether any difference existed histologically or ultrastructurally between the two diseases and (ii) whether immunofluorescence studies demonstrated any differences and whether they gave clues to possible pathogenetic mechanisms involved.

2.1.2 GLOMERULAR CRESCENTS

In some cases of both WG and MPA crescents were seen within glomeruli. The precise origin of cells in crescents is unclear and the mechanism of their generation unknown. Many attempts have been made to determine the histogenesis of the cells found in glomerular crescents, both in animal models and in human biopsy material. Histological and ultrastructural observations have suggested an origin from the parietal epithelial cells of Bowman's capsule (Morita, Suzuki & Churg, 1973; Min et al, 1974;), though not excluding a role for monocytes (Magill and Wadsworth, 1982). Glomerular culture and histochemical methods have favoured a monocytic origin (Atkins et al, 1976; Holdsworth et al, 1980; Ferrario et al, 1985), while cytogenetic studies have shown that crescent formation cannot be explained entirely by macrophage migration (Schiffer and Michael, 1978). An experimental rabbit model of serum nephritis has demonstrated monocytic involvement (Cattell and Aldridge, 1981), but indicated that parietal cells are also present, particularly in the late stage of development of crescents (Cattell and Jamieson, 1978). More recently, monoclonal antibodies have been used as investigative tools and have suggested that, in cultured glomeruli, epithelial cells make little contribution (Handcock and Atkins, 1984). In frozen sections, use of a monoclonal antibody against cytokeratin intermediate

filaments, a constituent of epithelial cytoplasm (Sun, Shih and Green, 1979; Holthofer et al, 1983), suggested that epithelial cells contribute significantly to crescent formation (Magil, 1985), although macrophages were also detected by non-specific esterase histochemistry.

The histological appearance of crescents varies from purely cellular to completely fibrous. Fibrocellular crescents have been shown to posses an antigenic determinant in common with renal tubular cells and natural killer cells, whereas the cells of cellular crescents appeared to bear mainly macrophage markers (Nolasco et al, 1984). This is supported by the work of Cattell and Jamieson (1978) on an experimental rabbit model.

This section investigated the differentiation of cells in glomerular crescents in paraffin embedded human renal biopsies, including four cases of WG, by the use of monoclonal antibodies against cytokeratin, leucocyte common antigen and epithelial membrane antigen, and of a polyclonal antibody against muramidase, a marker of monocytes/macrophages (Mason and Taylor, 1975). This last antibody has previously been used to investigate the significance of monocytes in normal kidney (Marshall and MacIver, 1984).

2.1.3 MAST CELLS

Although the aetiology of WG is uncertain some patients have raised serum IgA (Fauci et al, 1985) and others have a raised serum IgE (Conn et al, 1976; Singh, Yap and Douglas, 1982). Circulating immune complexes are sometimes found (Fauci et al, 1983; Conn et al, 1976; Hu, O'Laughlin and Winkelmann, 1977) and some renal biopsies show deposition of immunoreactants within the glomeruli (Fauci and Wolff, 1977). The success of antimicrobial therapy in some cases of WG has led to the suggestion that a hypersensitivity response to an underlying infection may trigger the disease (De Remee, McDonald and Weiland, 1985).

The interstitial inflammatory infiltrate in renal biopsies in WG is predominantly T cell (Berge et al, 1985). This is not inconsistent with the hypothesis of increased hyperactivity to inhaled antigens or pathogens (Douglas et al, 1976; Godman and Churg, 1954).

Although immune complexes are not always found in glomeruli from patients with MPA , there are well documented associations of MPA with exogenous antigens, especially hepatitis B, and the vasculitis is thought to result from immunoglobulin deposition in the vessel wall (Cupps and Fauci, 1981). No such associations have been recorded for WG.

In view of the possible implications of the role of IgE and

mast cells in WG and MPA by precipitating vascular injury , we investigated a series of renal biopsies to find the distribution of deposited or cell-associated IgE and compared them with the findings in renal biopsies from patients with MPA and diffuse endocapillary glomerulonephritis.

2.1.4 NASAL BIOPSY

Although the syndrome of classical WG is a triad of necrotising granulomatous vasculitis of lung and upper respiratory tract and a focal and segmental necrotising glomerulonephritis (Cupps and Fauci, 1981) patients often present to otolaryngologists or respiratory physicians with non-specific signs and symptoms of a systemic inflammatory disease, as well as complaints of sinusitis, nasal ulceration and discharge, or haemoptysis (Fauci and Wolff, 1983). On some occasions renal failure may precipitate presentation (Caralis, Falanga and Pardo, 1981) and up to 85% of cases at presentation will have renal involvement, albeit asymptomatic even in so-called limited WG (Fauci et al, 1983): since it is important to diagnose WG rapidly renal biopsy has been performed in some centres routinely. Its value has been questioned because biopsy appearances are frequently non-specific (Leavitt and Fauci, 1986), showing anything from minimal glomerular changes through focal and segmental glomerular lesions to crescentic glomerulonephritis with a small vessel vasculitis. We studied 19 patients where both renal biopsy and respiratory tract biopsy had been performed as a part of the clinical evaluation. The contribution of renal biopsy to diagnosis and prognosis was assessed.

2.2 MATERIALS AND METHODS

2.2.1 Histological Assessment of Renal Biopsies

Selection of Patients: Both the WG and MPA groups consisted of patients who had presented at Edinburgh hospitals over the period 1968-1987. Patients were allocated according to the working clinical diagnosis supplied by a clinician based on biopsy findings, clinical assessment , and response to therapy. All patients allocated to the WG group had clinical evidence of upper and/or lower respiratory tract involvement, and most had biopsy evidence confirming the clinical suspicion. None of the cases included had clinical features of atypical disease.

Using these criteria 26 biopsies from 26 cases of MPA were included, and 23 biopsies from 22 patients with WG. A brief clinical summary was made for each patient.

Light Microscopy: Paraffin sections of buffered formalin-fixed tissue were stained with haematoxylin and eosin, periodic acid-Schiff, and Martius Scarlet Blue.

Immunofluorescence: Cryostat sections were examined using antisera to IgG, IgM, IgA, fibrin, complement C1q, C3, C4, and immunoglobulin light chain kappa and lambda (Scottish Antibody Production Unit, Carluke, Scotland). Standard techniques were employed (Harrison, Thomson and MacDonald, 1986).

Electron Microscopy: Tissue was available from 11 cases of WG and 14 of MPA. Tissue was fixed in gluteraldehyde, post-fixed with osmium tetroxide, stained with uranyl acetate and embedded in Araldite resin. Sections (40nm thick) were examined on Corinth 275B or Jeol S200 electron microscopes.

2.2.2 Renal Biopsies in the Diagnosis of WG

Three cases did not have respiratory tract biopsies. To assess the value of routine renal biopsy in the diagnosis of WG 19 patients were included , in whom diagnosis had been made by clinical assessment including histopathological findings. These patients had originally presented at otolaryngological, respiratory or renal clinics. A summary was made of clinical records. Percutaneous renal biopsies were dealt with as described. Nasal or lung biopsies were routinely fixed in formalin for paraffin sections. Tubular atrophy in biopsies was scored on four point scale where 0 was no atrophy, 1 was minimal atrophy, 2 was moderate diffuse or severe focal atrophy, and 3 was severe atrophy.

2.2.3 Glomerular Crescents

Selection of Cases for Inclusion in the Study of Glomerular Crescents

For a diagnosis of crescentic glomerulonephritis it was

necessary for at least 70% of glomeruli to possess crescents. Control renal tissue was obtained from five nephrectomy specimens, removed because of renal carcinoma: none of these showed any significant glomerular or tubulo-interstitial lesion. Four cases of crescentic glomerulonephritis with an underlying focal and segmental necrotising glomerulonephritis as a result of WG had sufficient paraffin-blocked material to be included in the study. A further 14 cases showing a crescentic glomerulonephritis were also studied. The underlying diagnoses of these other cases of crescentic glomerulonephritis, were anti-glomerular basement membrane disease, (on the basis of linear immunofluorescence staining) and diffuse endocapillary proliferative glomerulonephritis. In six cases no underlying diseases was established.

Reagents and Techniques

The antibodies used were a polyclonal anti-muramidase raised in rabbit (Dako Antimuramidase, Dako Ltd, High Wycombe, UK) (Mason and Taylor, 1975) and mouse monoclonal antibodies against epithelial membrane antigen (EMA), which is a surface marker on many epithelial cells (Dako EMA, Dako Ltd) (Heyderman, Steele and Ormerod, 1979), against cytokeratin (CCK), which is a cytoplasmic intermediate filament (Dako PKK1, Dako Ltd.) (Holthofer et al, 1983),

and against leucocyte common antigen (LCA), which is present on the surface of most lymphoid and many monocytic cells (Dako LC, Dako Ltd.) (Dalchau, Kirkley and Fabre, 1980). Immunoperoxidase methods were employed, and were the standard techniques for paraffin sections (Sternberger et al, 1970; Salter, Krajewski and Dewar, 1985), sections being trypsinized before incubation with the primary antisera.

Assessment

The number of crescents per case was recorded and ranged from 2 to 24 (Table 1). The cells staining with the antibodies were noted and a count of the number of LCA positive cells in the glomerular tuft and the crescents was made. Cytokeratin positivity in the crescent was graded on a four point scale (0-3) where 0 indicated no positive staining, and 3 signified that more than 50% of the crescent cells were positive. No attempt was made to quantify EMA and muramidase positivity, but their distribution was noted. Assessment was made by two observers and was found to be readily reproducible.

2.2.4 IgE in Renal Biopsies

Renal tissue from a variety of conditions was investigated for the presence of IgE. Nine cases of WG (seven male, two female; median age 49 years, range 19-63 years), and nine

of MPA (seven male, two female; median age 56 years, range 30-69 years) were included, as well as six cases of proliferative glomerulonephritis (Four male, two female; median age 30 years, range 18-46 years. Initial renal function, as assessed by blood urea, was not significantly different between the WG and MPA groups (WG urea 21.8 $\pm$ 6.4 mmol/l; MPA were 21.9 $\pm$ 5.5 mmol/l). In addition, blocks from four normal kidneys were studied as controls.

Percutaneous renal biopsies were processed routinely and in addition an assessment of the degrees of inflammation (using a semiquantitative scale 0-4) was made by two independent observers. A score of 0 was assigned when no inflammation was seen, 1 when there was minimal inflammation, 2 when a diffuse infiltrate or aggregates of inflammatory cells were seen, 3 when up to half the interstitium consisted of inflammatory cells, and 4 when areas of inflammation became confluent and were associated with severe tubular atrophy. The use of renal biopsies rather than nasal biopsies avoided the complication of non-specific inflammatory change related to secondary infection. Polyclonal rabbit anti-human IgE (Dako Ltd) was used in an immunoperoxidase system to localise IgE.

No IgE-containing immune complex deposits were identified. Two types of cell staining were seen. Plasma cells were recognised morphologically and by cytoplasmic staining for IgE. Mast cells were recognised by staining of the

cytoplasmic membrane for IgE. Because percutaneous biopsies were used there was insufficient tissue to permit an accurate quantitative assessment such as that used by Colvin and colleagues (1974) and so mast cells were scored according to whether or not the cells were solitary or associated with other inflammatory cells. The number of mast cells in each biopsy and the proportion of these cells associated with areas of inflammation was recorded. All renal biopsies studied contained renal cortex.

2.3 RESULTS

2.3.1 Comparison of Renal Biopsies from WG and MPA

Clinical

The WG group comprised 22 patients (16M:6F) with a mean age [range] of MPA group comprised 26 patients (16M:10F) with a mean age [range] of 52 years [13-73 years]. Apart from the absence of upper respiratory tract lesions in patients with MPA there was no difference between the two groups in terms of general symptomatology and evidence of multi-organ involvement. Three patients from each group had low levels of circulating immune complexes and all patients had a raised erythrocyte sedimentation rate. Renal function, as assessed by blood urea, creatinine and creatinine clearance was worse in the MPA patients.

Light Microscopy

Results are summarised in Table 2.3.1 for WG. Thirteen of 23 biopsies had evidence of a focal and segmental glomerulonephritis, and only three biopsies from patients with WG were normal. In only one case was active vasculitis seen; this was in an afferent arteriole. Associated with the glomerular changes there was an active chronic interstitial nephritis of variable degree. Although some eosinophils were present they were never the predominant inflammatory cell type.

All the cases of MPA were classifiable as microscopic form; that is, there was a focal and segmental necrotising glomerulonephritis with no evidence of major vessel involvement. Only one case showed an active vasculitis in an arteriole and approximately one third of cases had significant crescent formation. Most cases had an active interstitial nephritis and this tended to be more severe than in the WG biopsies, in keeping with clinical assessment of renal function. It was noted in this regard that biopsies from WG patients were obtained as part of the diagnostic assessment of systemic disease even if there was no overt renal disease whereas in MPA renal biopsy was usually prompted by deterioration of renal function. Cases of MPA tended to have a higher proportion of eosinophils in the inflammatory infiltrate, but this was of no diagnostic significance when semiquantitative scores where compared. One case of MPA had a marked venulitis but this was never observed in WG.

Immunofluorescence

This was available in 22 WG biopsies and 26 MPA. In 73% of WG biopsies and 86% of MPA biopsies there was evidence of deposition of immunoglobulins; but in all cases this was in small amounts. Complement was present in 59% of WG and 52% of MPA, again in small amounts. Deposited material was almost invariably glomerular and was seen in mesangium and/or basement membrane. Only 18% of WG and 14% of MPA

biopsies had no evidence of complement or immunoglobulin deposition. IgG was the most frequently present immunoglobulin, (present in 45% of WG and 38% of MPA). Fibrin was associated with areas of necrosis and crescent formation.

EM Results

The EM results in the two groups were broadly similar and showed no distinguishing features at the ultrastructural level (Table 2.3.2) (Figures 2.3.1 - 2.3.8).

(i) Glomerular Basement Membrane: Intramembranous deposits were seen in both conditions. In five cases of WG and one of MPA these deposits were pale and diffuse but small, dense, discrete masses in the GBM were also observed in three cases of MPA. Subendothelial deposits were seen in five cases of WG and one case of MPA but subepithelial deposits were rare in both conditions, being seen in only one case of each.

A rarefied zone of variable thickness containing scattered fibrils was seen on the endothelial aspect of the GBM in 4 cases of MPA and 2 cases of WG, although focal thickening of the GBM was observed in only one case of MPA. Breaks in the GBM were very rare, being seen in only one case in

each of the two conditions and no holes in the GBM were seen in either group.

(ii) Mesangium: An increase in mesangial matrix was found in over half the cases studied in both groups but cellular proliferation was observed in only three cases of MPA and one case of WG Collagen fibres were seen in the mesangial regions in two cases of WG and one case of MPA. Mesangial deposits were seen in only one case of WG.

(iii) Epithelial Cells: Foot process (pedicel) fusion was observed in the majority of cases in both groups. Intracytoplasmic granules were found only once, in a case of WG.

(iv) Endothelial Cells: In over half of the cases in each group endothelial cell swelling was present.

(V) Capillary Lumina: Although single platelets were seen in the capillary lumina of four cases of WG and one case of MPA, clumps of platelets were observed in only one case of each. Neutrophils were present in three cases of MPA and in four cases of MPA intraluminal fibrin was observed.

TABLE 2.3.1:

Renal Histopathological Diagnosis by Light Microscopy in 23 biopsies from 22 Cases of Wegener's Granulomatosis

DIAGNOSIS	NUMBER
Focal and segmental necrotising glomerulonephritis ± superimposed crescentic glomerulonephritis	5
Focal and segmental necrotising glomerulonephritis	7
Focal and segmental proliferative glomerulonephritis	1
Diffuse mesangial proliferative glomerulonephritis	7
Histology normal	3
	23 TOTAL

TABLE 2.3.2 Summary of Electron Microscopy Findings in MPA and WG

		number of cases	
		MPA (n=14)	WG (n=11)
Mesangium	Cell proliferation	3	1
	Increase in matrix	8	7
	Presence of collagen	1	2
Endothelial cells	Cell swelling	9	8
Epithelial cells	Loss of pedicel structure	10	6
	Intracytoplasmic granules	0	1
GBM	Thickening	1	0
	Breaks	1	1
	Holes	0	0
	Subendothelial deposits	1	5
	Intramembranous deposits		
	(i) pale, diffuse	1	5
	(ii) dense, discrete	3	0
	Subepithelial deposits	1	1
	Rarefied sub-endothelial zone	4	2
Capillary lumina	Platelets - single	1	4
	Platelets - clumps	1	1
	Fibrin	4	0
	PMN	3	0

FIGURE 2.3.1 A break in the GBM in an MPA case. (x 23,900)

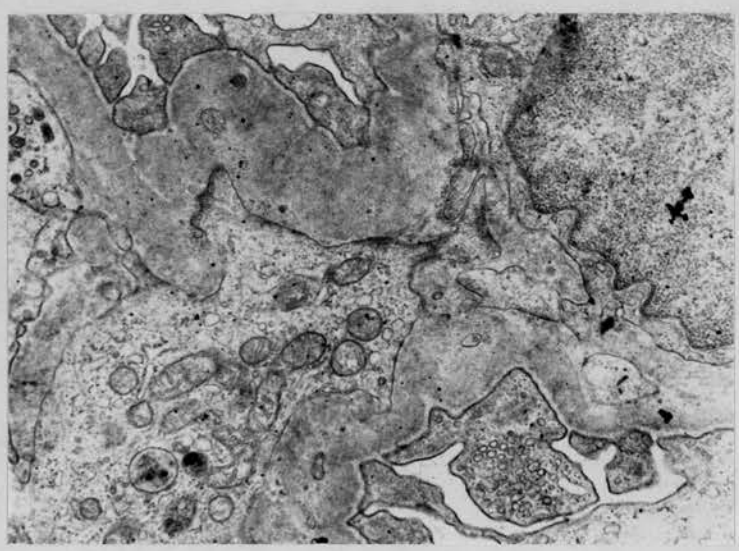


FIGURE 2.3.2 MPA case showing pale, diffuse and dense discrete intramembranous deposits. (x 25,000)

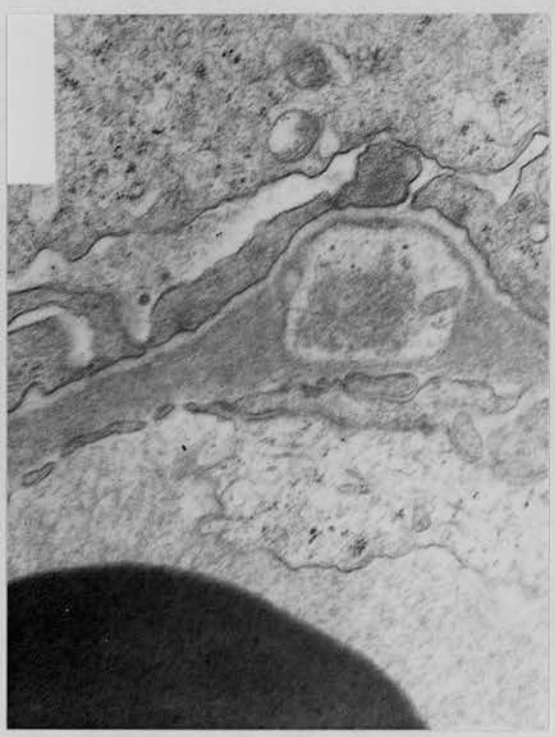


FIGURE 2.3.3 An intramembranous deposit in a case of WG. The vacuolated appearance is caused by epithelial cytoplasmic surrounding the deposit.
(x 30,000)

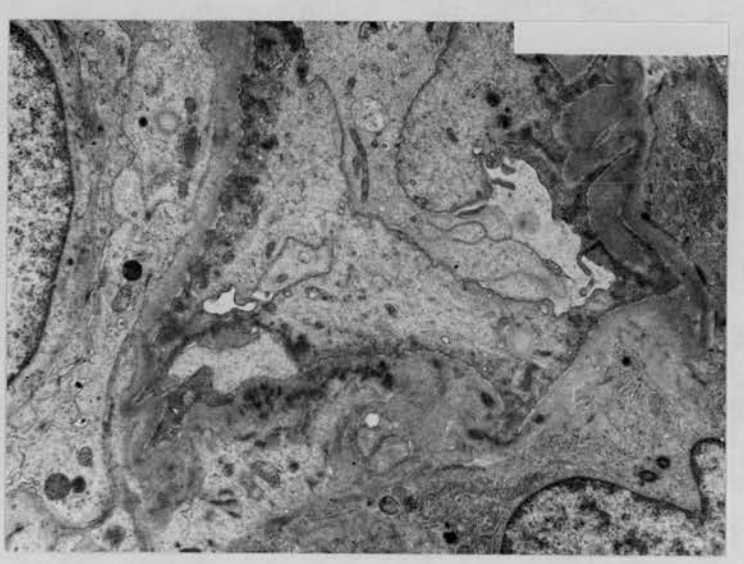


FIGURE 2.3.4 WG case showing epithelial foot process fusion and a small subepithelial deposit surrounded by mesangial cell processes. (x 7,000)

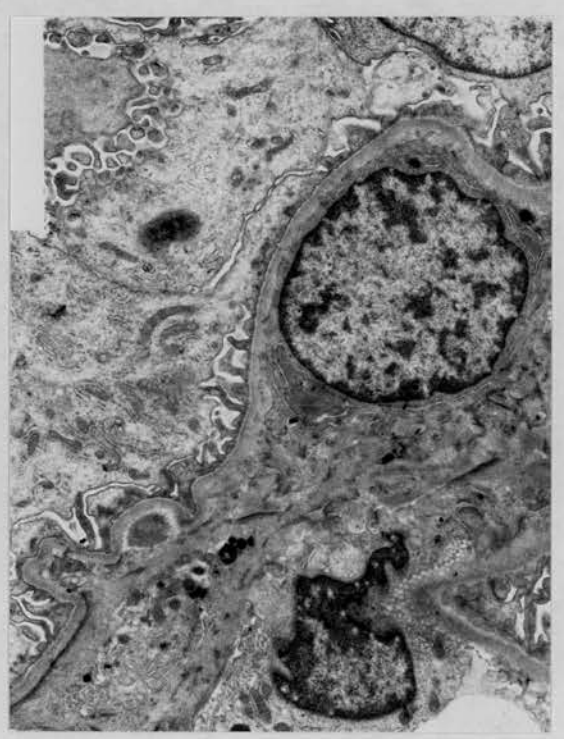


FIGURE 2.3.5 WG case showing deposits which are partially subendothelial and partially intramembranous. Foot process fusion is also seen.
(x 7,000)

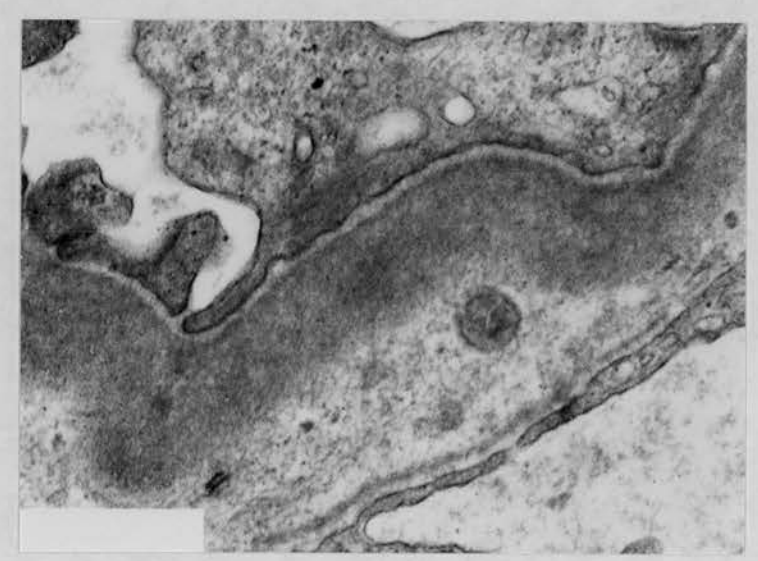


FIGURE 2.3.6 Rarefied subendothelial zone suggestive of fibrin deposition, containing a small deposit in a case of MPA. Loss of pedicel structure is also seen.
(x 35,000)

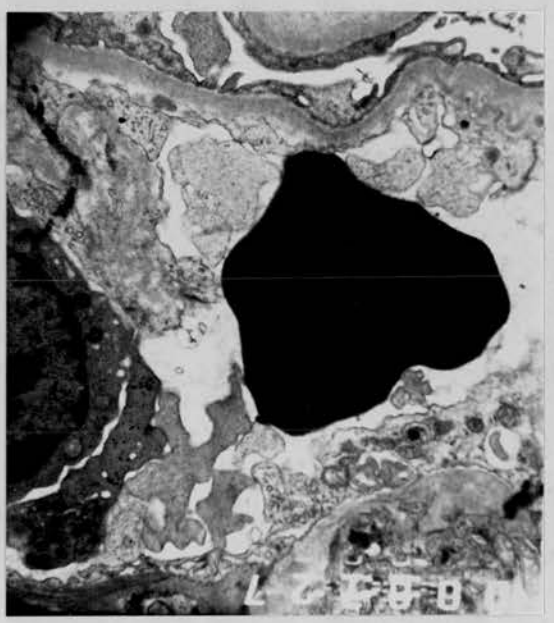


FIGURE 2.3.7 MPA case showing a subendothelial deposit and epithelial foot process fusion.
(x 12,000)

FIGURE 2.3.8 WG case showing fibrils probably of collagen in the mesangium.
(x 11,700)

2.3.2 Results of the Study : the Contribution of Renal Biopsy to diagnosis of WG

Clinical Features: See Table 2.3.3

There were 16 male and 6 female patients with a median age [range] at presentation of 45 years [19-68y]. The median [range] duration of symptoms before presentation was 6 months [1-60m]. Seventeen patients had presentation symptoms related to upper and/or lower respiratory tract disease. In addition 2 cases had significant cutaneous involvement and in 4 cases part of the initial symptomatology was due to severe renal failure. Many patients had haematuria and proteinuria. Only 2 urine specimens from 16 patients examined microscopically were normal. At presentation 10 of 16 chest radiographs were abnormal, showing diffuse non-specific shadowing in 5, consolidation of a single lobe in 3, and cavitating lung lesions in 2. Nine patients had normal blood urea at presentation (< 7mmol/1) and 10 had raised blood urea (>7 mmol/1).

All patients received cytotoxic drug therapy and the 1year survival was 82%. In 17 cases followed up for at least 1 year, 5 have died, 1 who had a normal blood urea at outset (1 out of 8 patients), and 4 who had a raised blood urea (4 out of 9 patients). Death was caused by septicaemia in 3 cases, gastrointestinal haemorrhage and renal failure in 1 case, and pulmonary embolus in the context of active

widespread vasculitis despite cytotoxic drug therapy in 1 case.

Pathological Findings

Sixteen patients had nasal biopsies, and a firm or strongly suspected diagnosis of WG was made in 8 of these because of the presence of a necrotising vasculitis, multinucleate giant cells, granuloma formation, and florid chronic inflammation. In the remaining biopsies no specific features suggestive of WG were identified. Two patients had transbronchial lung biopsies and one of these showed a necrotising granulomatous vasculitis. Another patient had a lung biopsy in addition to a nasal biopsy and this showed legionella pneumonia. Four of the 19 renal biopsies showed a crescentic glomerulonephritis, 5 showed a focal and segmental necrotising glomerulonephritis, 4 had a focal and segmental proliferative or sclerosing glomerular lesion, 4 had a diffuse mesangial proliferative glomerulonephritis, and only 2 were histologically normal. Respiratory tract and renal biopsy findings are summarised in Table 2.3.4.

When biopsies were scored for tubular atrophy on a 0-3 scale it was found that the average score for biopsies from cases with normal blood urea at presentation was 0.9, compared with 1.9 for cases with raised blood urea.

TABLE 2.3.3

SUMMARY OF CLINICAL DATA OF 19 PATIENTS WITH

WEGENER'S GRANULOMATOSIS

Patient	Age (Y)	Sex	Length of Prodromal Illness (months)	Initial Urea (mmol/l)	Presenting Symptoms	Chest X-ray	Urine Analysis
1	23	M	3	50	Haemoptysis URT*	Bilateral infiltrates	RBC casts Proteinuria
2	50	F	10	4	URT	Nad+	Nad
3	45	M	20	8	URT	Nad	RBC casts Proteinuria
4	39	M	5	3	URT Pyoderma	Nad	Haematuria Proteinuria
5	24	F	1	5	Arthritis Skin vascu-litis	Nad	Not available
6	56	M	36	12	URT	Not available	Haematuria Granular Casts
7	64	M	11	10	URT	Not available	Proteinuria WBC and hyaline casts
8	31	M	3	9	Haemoptysis Cough	Right lower consolid-ation	Proteinuria Micro-haematuria
9	22	M	1	4	Haemoptysis URT	Nad	Granular casts
10	43	M	6	30	URT Renal failure	Right shadowing	Proteinuria WBC casts

continued overleaf

Patient	Age (Y)	Sex	Length of Promromal Illness (m)	Initial Urea (mmol/l)	Presenting Symptoms	Chest X-ray	Urine Analysis
11	54	F	24	5	URT	Diffuse increased markings	Nad
12	68	M	60	7	URT	Nad	RBC casts
13	57	F	5	30	URT	Right pleural effusions Atelectasis	Haematuria RBC cases
14	39	M	6	5	Haemoptysis URT	Not available	Haematuria
15	19	M	3	3	URT Haematuria	Cavitating lesions right lung	Haematuria Proteinuria Proteinuria
16	63	M	1	22	Pyrexia Renal failure	Non-specific shadowing right base	Anuric
17	54	F	6	3	Haemoptysis Pleuritic pain	Cavitating lung lesions	Haematuria
18	39	M	1	25	Cough Haemoptysis Renal failure URT	Consolid-ation	Haematuria
19	54	M	2	4	URT Pulmonary embolus	Consolid-ation	Nad

* URT: Upper Respiratory Tract

+ Nad: No Abnormality Detected

TABLE 2.3.4

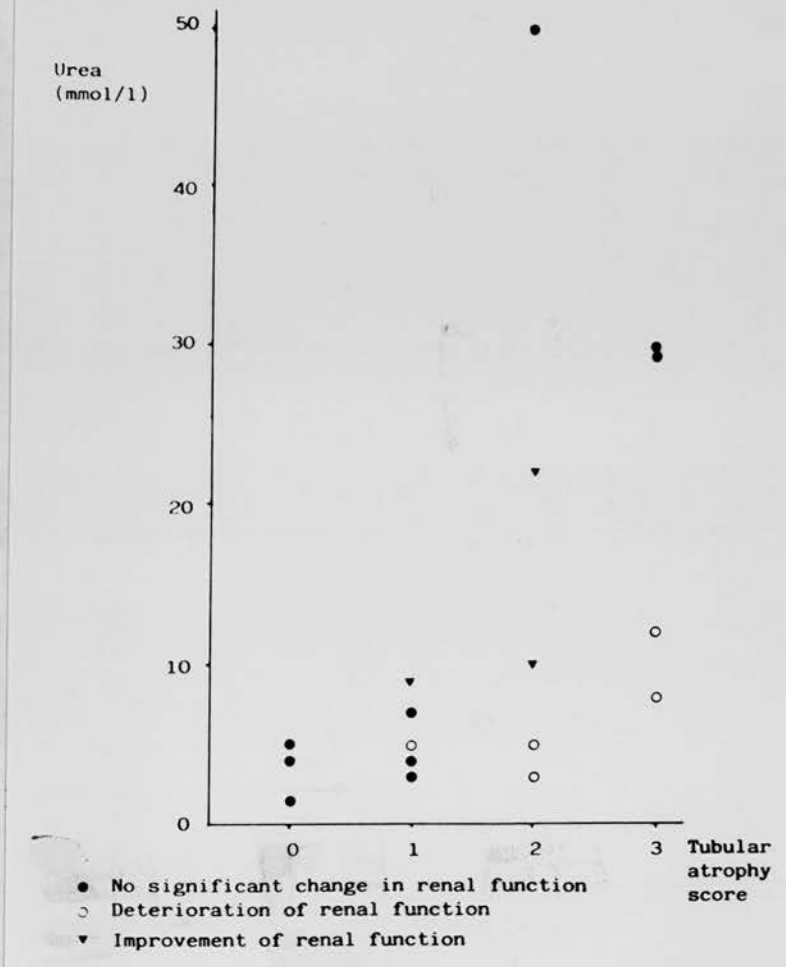
SUMMARY OF RENAL AND RESPIRATORY TRACT : Pathology

Nasal/Transbronchial Biopsy	Renal Biopsy	Number of Cases	
very suspicious of WG	Segmental necrotising GN	5	
	Segmental sclerosis/ proliferation	0	
	Non-specific/normal	4	
Non-specific active chronic inflammation	Segmental necrotising GN	3	
	Segmental sclerosis/ proliferation	5	*
	Non-specific/normal	2	

* Two of these cases had > 70% crescents

FIGURE 2.3.9

This shows the presentation urea plotted with the degree of tubular atrophy scored on the renal biopsy from no significant atrophy (score 0) to severe tubular atrophy (score 3). Also recorded is the direction of change of the urea concentration on follow-up.



2.3.3 Results of Investigation into the Differentiation of Glomerular Crescents

Control Kidney

Muramidase

This was distributed in the cytoplasm of cells of most convoluted tubules, in monocytes and in neutrophils. Glomerular capillaries contained an average of 5.5 intraluminal positive cells per tuft (range 0-18).

Epithelial Membrane Antigen

The cytoplasmic membrane of cells of collecting tubules and many convoluted tubules reacted positively. Occasional glomeruli contained very small foci of positive staining, as previously described by Howie (1986).

Cytokeratin

The cytoplasm of cells of collecting tubules were strongly positive and many convoluted tubules also reacted, but some parietal epithelial cells of Bowman's capsule did react strongly (mean 32%, range 0-100%) Figure 2.3.10.

Leucocyte Common Antigen

Monocytes, interstitial and intravascular lymphocytes reacted and each glomeruli tuft contained an average of three positive intraluminal cells (range 0-9). No renal

elements and, in particular , no cells in mesangial regions reacted.

Crescentic Glomerulonephritis

Significant results are summarized in Table 2.3.5. Crescents were also classed as being cellular, fibrocellular or fibrous.

Muramidase

The anti-muramidase antibody reacted with convoluted tubules, some capsular parietal epithelial cells in glomeruli in which crescents were not present, many intra- and extravascular polymorphs and macrophages, and many crescent cells. The reaction of crescent cells was not uniform; a few cells reacted strongly, more reacted weakly, but most were negative. Quantification was not attempted.

Epithelial Membrane Antigen

The antibody reacted with some convoluted tubules and collecting tubules but no crescents, with the exception of three cells in a glomerular crescent in one case.

Cytokeratin

Overall, 34% of crescents contained cytokeratin (CK) positive cells. The median grade of positive crescents

with reacting cells was grade 1 (mean 1.5). Fibrous crescents did not react with the antibody, and in epithelial and fibroepithelial crescents the intensity of reaction varied considerably within a single crescent (Fig 2.3.11). Crescents with marked mitotic activity tended to have a higher proportion of positive cells, although it was not possible to determine the nature of the mitotic cells. It was noted that on occasions CK positive cells were concentrated on the aspects of the crescent adjacent to the glomerular tuft.

Leucocyte Common Antigen

Glomerular capillaries contained an average of 1.7 positive cells per glomerulus and these were all intraluminal (range 0-11). Thirty percent of crescents contained positive cells (mean 1.1 cell per crescent) and these tended to be in the outer part of the crescent. In eight cases, positive cells in the crescent were associated with a ring of positive cells lying just outside Bowman's capsule suggesting that the intracrescentic cells may have entered through Bowman's capsule (Fig 2.3.12).

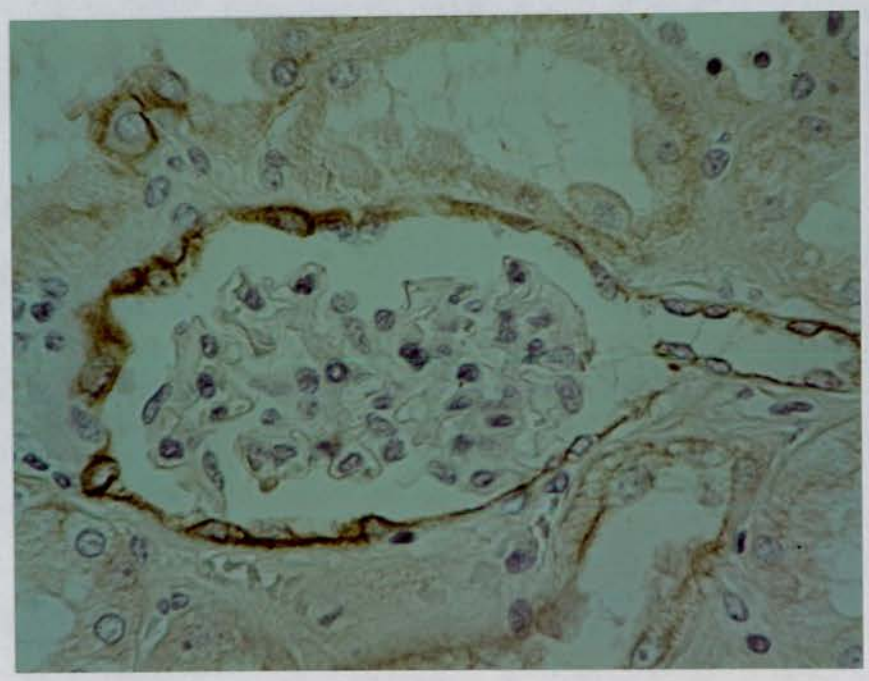


FIGURE 2.3.10 A normal glomerulus showing a positive reaction for cytokeratin by most parietal epithelial cells. Note the variation in reaction intensity. PAP x 500.

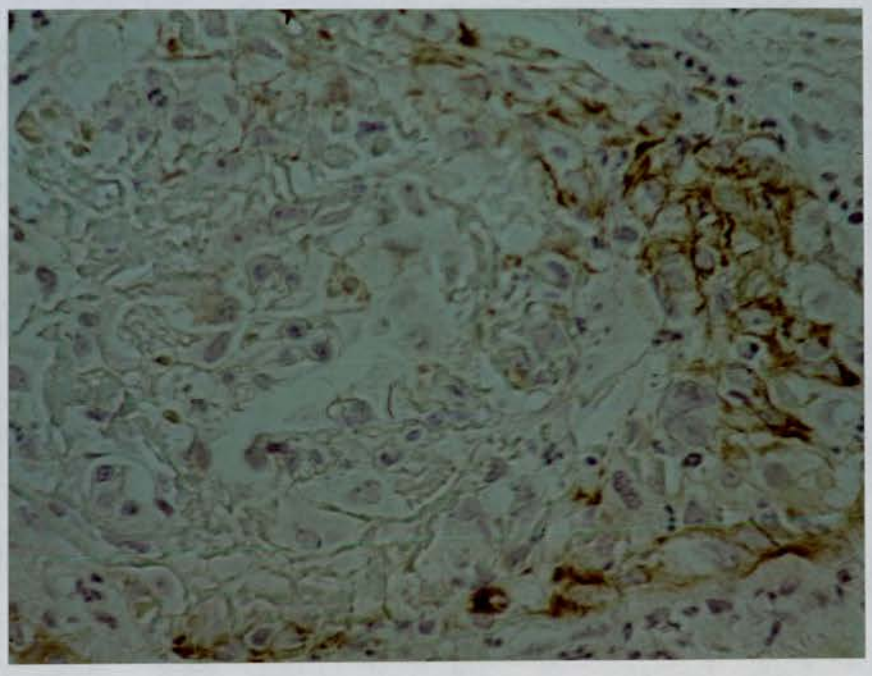


FIGURE 2.3.11 A glomerulus containing a cellular crescent which is strongly positive for cytokeratin. The glomerular tuft does not react but some nearby tubular cells are positive. PAP x 320.

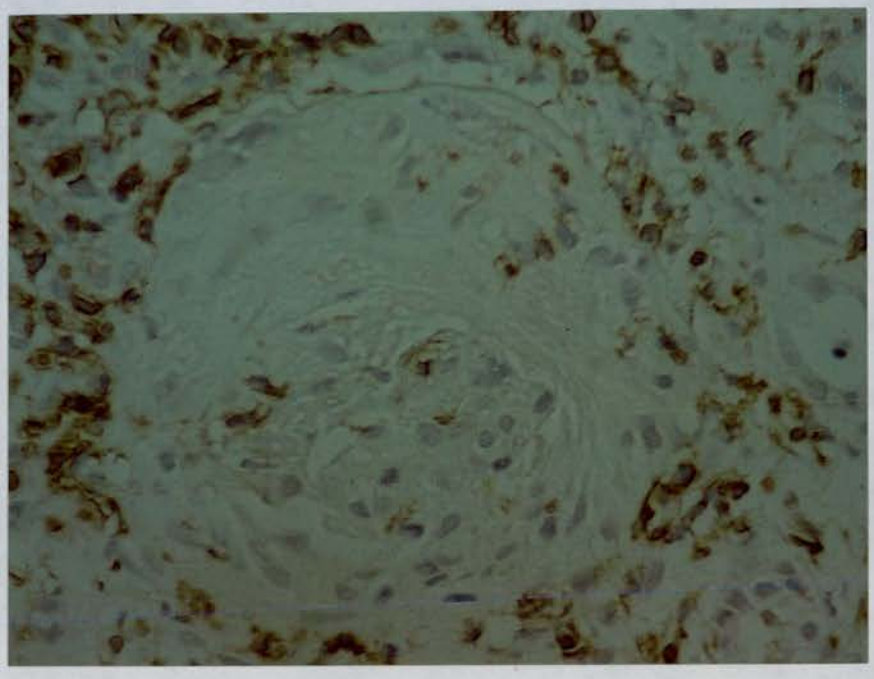


FIGURE 2.3.12 A fibrocellular crescent, compressing the glomerular tuft, surrounded by numerous leucocyte common antigen positive mononuclear cells. Some reacting cells are present in the crescent and also in the lumina or tuft capillaries. PAP X 320.

TABLE 2.3.5

Tabulation of crescentic glomerulonephritis (CGN) cases giving diagnosis associated with CGN, the number of crescents examined, the number of glomeruli containing crescents with varying grades of cytokeratin positivity and the average number (and range) of leucocytes in tuft capillaries and crescents. Cases 1, 6, 16 and 18 were of WG.

Case No		No of Crescents	Grade of Staining* 0	1	2	3	Capillary Tuft Leucocytes	Crescent Leucocytes
1	FSN	13	8	5	0	0	2.4 (1-5)	2 (0-5)
2	NL	13	6	4	3	0	0.7 (0-5)	0.5 (0-3)
3	NL	4	3	1	0	0	2.5 (0-5)	No tissue
4	NL	10	9	0	1	0	0.4 (0-2)	0
5	aGBM	8	8	0	0	0	0.4 (0-2)	0.3 (0-2)
6	FSN	13	8	0	2	3	5.5 (2-11)	2.0 (0-6)
7	NL	9	2	3	3	1	2.0 (0-5)	3.0 (0-9)
8	DEPGN	9	9	0	0	0	0.3 (0-2)	0
9	DEPGN	9	7	2	0	0	2.8 (1-3)	0.3 (0-1)
10	GBM	2	Insufficient tissue				5.0 (1-2)	6.0 (6)
11	aGBM	3	2	0	1	0	2.5 (105)	No tissue
12	aGBM	7	7	0	0	0	1.4 (0-4)	0.1 (0-1)
13	NL	6	5	1	0	0	1.0 (0-2)	0
14	NL	3	3	0	0	0	0.7 (0-1)	0
15	aGBM	5	Insufficient tissue				1.8 (0-4)	0.9 (0-2)
16	FSN	3	2	0	1	0	1.6 (0-3)	No tissue
17	aGBM	9	8	0	1	0	1.9 (0-5)	0.9 (0-3)
18	FSN	24	7	11	6	0	1.2 (0-3)	1.5 (0-4)

FSN = Focal and segmental necrotising glomerulonephritis (WG);

NL = No obvious lesion;

aGBM = Anti-glomerular basement membrane disease;

DEPGN = Diffuse endocapillary proliferative glomerulonephritis;

* 0 = No positive staining;

* 3 = More than 50% crescent cells positive.

2.3.4 Results of Investigation of IgE

General

Biopsies were scored for inflammation by two independent observers on two separate occasions and results were never more divergent than one grade on the 5 point scale. The median scores were 2.0 (WG), 2.0 (MPA), 1.5 (proliferative glomerulonephritis) and 1.0 (control) respectively. There was no significant difference between the WG and MPA groups.

Cytoplasmic IgE Plasma Cells

These were infrequent and were always associated with other inflammatory cells (Fig 2.3.13).

Surface IgE-Bearing Mast Cells

Normal renal tissue contained very infrequent surface IgE-bearing mast cells in close proximity to peritubular capillaries and the vascular pole of glomeruli. In proliferative glomerulonephritis, WG and MPA there was an increased number of mast cells demonstrated by anti-IgE immunoperoxidase (Figure 2.3.14), compared to controls, although the frequency of mast cells varied widely between biopsies (Table 2.3.6). It was also noted that the intensity of staining varied widely, even within the same biopsy. As expected, the proportion of mast cells

associated with an inflammatory cell infiltrate increased as the degree of inflammation increased. However, at each grade of inflammation the proportion of mast cells found in association with inflammatory cells was lower in WG group than in the MPA and proliferative glomerulonephritis groups (Table 4.1), indicating that WG mast cells were more often unassociated with areas of inflammation. This difference between WG and MPA was statistically significant ($P < 0.02$), Mann-Whitney U Test, two-tailed) (Figure 2.3.15).

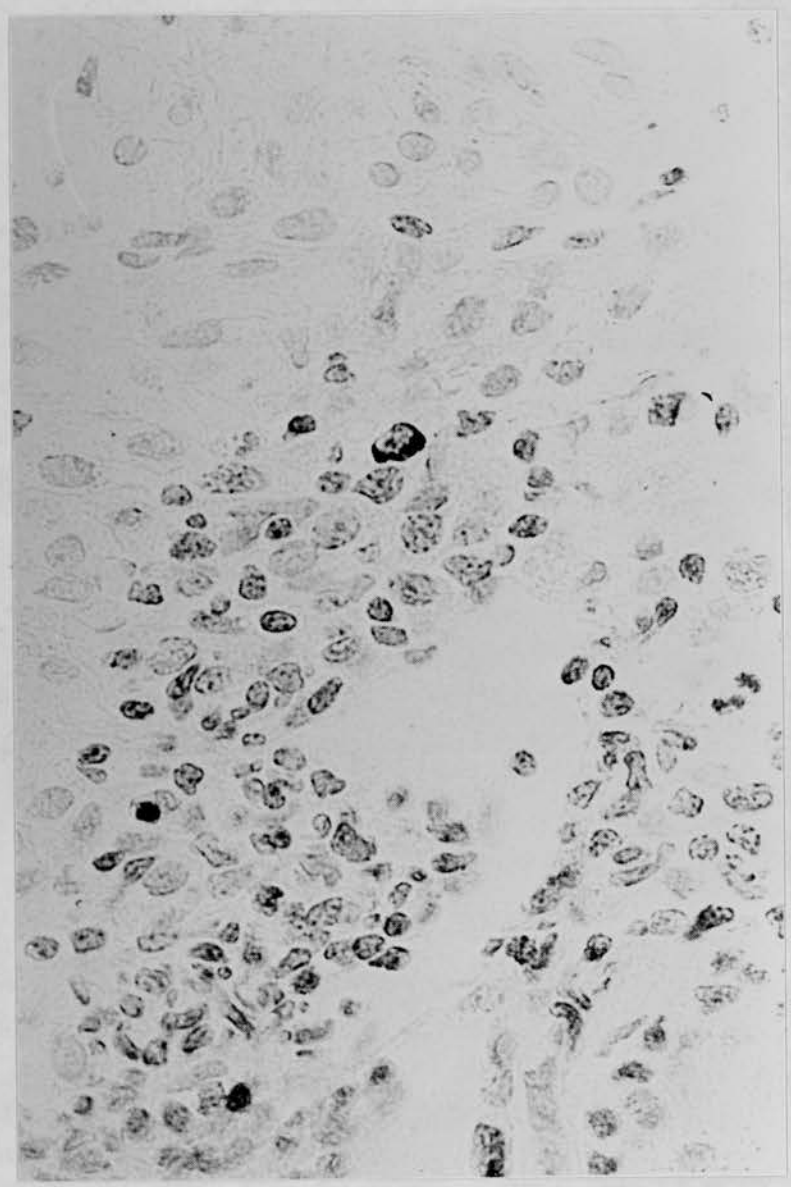


FIGURE 2.3.13 Group of mixed inflammatory cells with one plasma cell showing cytoplasmic staining for IgE (Case of microscopic polyarteritis). X 225.

FIGURE 2.3.14 Mast cells in a peritubular distribution with a rim of surface IgE stained by immunoperoxidase and unassociated with other inflammatory cells (Case of Wegener's Granulomatosis). X 225.

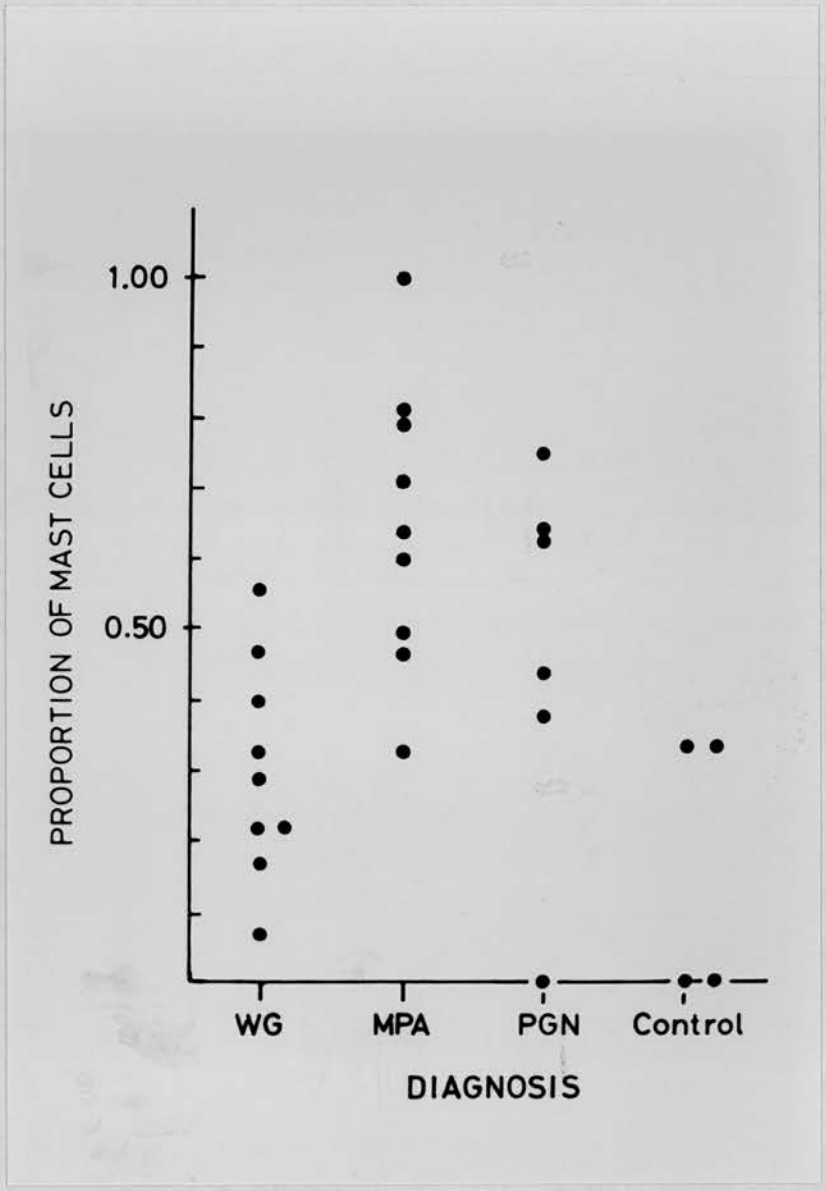


FIGURE 2.3.15 Proportion of mast cells in areas of inflammation for each of the diagnostic groups. WG, Wegener's Granuloma; MPA, microscopic polyarteritis; PGN, proliferative glomerulonephritis. The difference between the Wegener's Granuloma and microscopic polyarteritis groups is significant ($P < 0.02$; Mann-Whitney).

TABLE 2.3.6

The number of mast cells present in renal biopsies and the proportion in areas of inflammation grouped according to the grade of inflammation and diagnosis.

Grade of	WG		MPA		PGN		Control	
Inflammation	i/N	(P)	i/N	(P)	i/N	(P)	i/N	(P)
0	-		-		-		0/3	(0.00)
1	2/7	(0.29)	18/38	(0.47)	9/12	(0.75)	5/15	(0.33)
	7/42	(0.17)			0/1	(0.00)	1/3	(0.33)
	2/28	(0.07)			3/8	(0.38)	0/25	(0.00)
2	5/15	(0.33)	1/2	(0.50)	7/11	(0.64)	-	
	16/74	(0.22)	5/7	(0.71)	11/25	(0.44)		
			6/10	(0.60)	7/12	(0.63)		
			2/2	(1.00)				
			1/3	(0.33)				
3	2/9	(0.22)	41/64	(0.64)				
	31/66	(0.47)						
4	6/15	(0.40)	35/43	(0.81)				
	54/96	(0.56)	19/24	(0.79)				

i, mast cells in inflamed areas; N, total number of mast cells; P, proportion of mast cells in inflamed areas; WG, Wegener's granulomatosis; MPA, microscopic polyarteritis; PGN, proliferative glomerulonephritis.

2.4 DISCUSSION

2.4.1 Renal Biopsies in WG and MPA

Examination of the renal biopsies in WG and MPA by light microscopy and conventional stains revealed no significant qualitative difference in the type of renal damage, nor was any pathognomonic feature of either identified. Focal and segmental glomerular necrosis, sclerosis or proliferation, mesangial cell proliferation, mesangial matrix increase, periglomerular and interstitial inflammation or fibrosis, and tubular atrophy were present in both conditions and have all been previously recognised (Paronetto et al, 1962; Davson et al, 1948; Whitaker et al, 1973; Heptinstall, 1983; Dunnill, 1984). In accordance with the findings of Novak and colleagues (1984) no granulomata were identified in renal biopsies. However in another study six of 15 cases of WG with severe renal involvement have been described as containing peri- or para-glomerular granulomata (Pinching et al, 1983). In terms of severity of lesions the MPA biopsies showed a greater degree of damage. This reflects the clinical severity of renal involvement at presentation, and also the fact that whereas renal biopsies were performed in all cases of MPA because of symptomatic renal involvement, in a proportion of WG patients renal biopsy was taken in the absence of renal symptoms either to confirm the systemic nature of disease or to include subclinical renal injury. Small amounts of

immunoreactants were identified in the majority of cases of both WG and MPA but there was no difference between the two. These results are comparable to those of Horn et al, (1974), Pinching et al, (1983), although some groups report that positive immunofluorescence is uncommon (Ronco et al, 1983; Novak et al, 1984).

The findings of electron microscopy are similar to those previously reported (Aldo et al, 1970; Horn et al, 1974; Weiss et al, 1984; Magil 1978) with no distinctive features being identified (Norton et al, 1968). The presence of small subendothelial, intramembranous and subepithelial deposits supports the involvement of immune complex deposition in the pathogenesis of the renal lesions in WG and MPA (Horn et al, 1974; Magil et al, 1978; Churg and Grishman, 1972). The paucity of deposits seen both by immunological and electron microscopic methods may reflect rapid clearance of complexes by mesangium (Ronco et al, 1983) or merely the age of the lesion (Cochrane et al, 1959). The rarification of the subendothelial zone seen in two cases of WG and four of MPA is consistent with fibrin deposition (Heptinstall, 1983). This study failed to confirm the findings of Weiss and colleagues (1984), Juncos and colleagues (1979) and Watanabe and colleagues (1983) that glomerular thrombosis and not immune complex deposition is the primary event in the pathogenesis of renal lesions in WG. Intravascular lysis of neutrophils

(Donald et al, 1976) was not observed in WG biopsies.

In summary conventional light microscopy, immunofluorescence and electron microscopy failed to show any significant qualitative differences between WG and MPA. The findings are consistent with an immune complex pathogenesis for both diseases, but do not help to elucidate why the kidney should be involved in both diseases, and why the rates of progression of disease and responses to therapy for WG and MPA are often different.

2.4.2 The Value of Routine Renal Biopsy in Diagnosis of WG

Our clinical findings are in accord with series in the literature in terms of age, sex ratio, clinical presentation, survival and cause of death (Cupps and Fauci, 1981). In a review of more than 100 cases of WG significant renal involvement was present clinically in more than 40% (De Remee et al, 1986).

Whilst only 9/19 (47%) of nasal or transbronchial biopsies were very suggestive of WG by themselves, the combination of respiratory tract and renal biopsy findings would have suggested the possibility of WG in a further 8/19 (42%) cases. This is because 3 of these cases with non-specific active chronic inflammation respiratory tract biopsies had a focal and segmental necrotising lesion in glomeruli and a

further 5 had a focal and segmental glomerular lesion, either proliferative or sclerotic.

The degree of renal tubular atrophy has been regarded as a useful prognostic indicator in other renal diseases, such as membranous glomerulonephritis (Stanziale et al, 1985).

Thus renal biopsy has been shown to provide additional evidence to suggest the diagnosis of WG because it can demonstrate the systemic nature of the inflammatory process even if the classical features are not present in tissue studied. The degree of renal tubular atrophy may be useful in assessing permanent renal damage at presentation. Renal biopsy should therefore be considered as a useful investigation when confronted with cases of possible WG.

2.4.3 The Histogenesis of Crescents

We have shown that a small proportion of crescent cells contain cytoplasmic cytokeratin, and are therefore likely to be epithelial in origin (Sun et al, 1979; Holthofer et al, 1983). Thirty-two percent of normal parietal cells have been shown to contain cytokeratin. This is consistent with epithelial cells of the glomerular crescent being derived from the parietal epithelium of Bowman's capsule. The presence of large numbers of cytokeratin negative cells in the crescent is in keeping with the intermittent demonstration of cytokeratin in the parietal epithelium of the normal Bowman's capsule. This supports the work of Magil (1985) using frozen material, and of those who have used electron microscopy to demonstrate an epithelial origin (Morita et al, 1973; Min et al, 1974; Magil and Wadsworth, 1982). Our findings are not in disagreement with previous studies on cultured glomeruli (Atkins et al, 1976; Holdsworth et al, 1980; Handcock and Atkins, 1984) which demonstrated the importance of monocytes, because these studies did not specifically investigate parietal epithelial cell involvement, but were more concerned with the identification of podocytes (Magil, 1985). The muramidase positive staining seen in some parietal epithelial cells in crescentic glomerulonephritis is difficult to explain. One may postulate that these cells either produce muramidase normally in small

quantities, with the enhancement of production in crescentic glomerulonephritis, or that muramidase is excreted into Bowman's capsule and reabsorbed leading to a non-specific reaction (Balazs and Roepke, 1966).

Leucocytes can be identified in crescents, as others have shown, by histochemical or immunohistochemical methods (Atkins et al, 1976; Holdsworth et al, 1980; Handcock and Atkins, 1984; Nolasco et al, 1984; Magil, 1985; Ferrario et al, 1985). These were found frequently in the outer part of the crescent associated with leucocytes outside Bowman's capsule, in which there are known to be breaks in crescentic glomerulonephritis (Bohman, Olsen and Peterson, 1974).

The controversy surrounding the histogenesis of crescents has not been completely resolved, but we have confirmed that both mononuclear leucocytes and epithelial cells are present. This is consistent with the simple proposition that crescents are fundamentally epithelial in origin, and that leucocytes are entering the crescents as part of the overall reactive inflammatory process.

2.4.4 Mast Cells in Renal Biopsies

Mast cells identified by metachromatic stains or electronmicroscopy are found only rarely in the normal kidney, although their numbers increase in chronic

inflammation and neoplasia (Pavane - Macalusco, 1960; Colvin et al, 1974).

In one study showing an increased number of mast cells in various inflammatory and vascular lesions, a quantitative assessment was made on biopsy and nephrectomy material (Colvin et al, 1974).

In some of these cases vacuolated cells, thought to be degranulated mast cells, were seen actually within tubular epithelium. We have identified mast cells by their binding of IgE, and failed to see any in an intraepithelial position.

The distribution of mast cells detected in our cases was similar for MPA and proliferative glomerulonephritis, the majority of mast cells being present as part of a general inflammatory cell infiltrate. In WG however, the pattern was different, the mast cells being identified predominantly without an associated inflammatory infiltrate, although still in the typical perivascular sites seen in normals. This suggests that the increase in mast cells in WG is a phenomenon preceding the inflammation, and may therefore be of some pathogenetic significance. It is unlikely that IgE immunoperoxidase demonstrates all mast cells, and the variability of staining intensity we have seen would support this view. There may be other mast cells present in the biopsies but

not in an activated state, with insufficient bound IgE to be detectable by this technique.

The association found between WG and raised serum IgE in some patients (Conn et al, 1976; Singh et al, 1982) has led to the suggestion that mast cells in WG may act in the development of a vasculitis by predisposing to increased vascular permeability and facilitating deposition of immune complexes in the vessel wall (Leavitt and Fauci, 1986).

We have no evidence that IgE production by plasma cells in the kidney is increased in WG, since comparable numbers of IgE plasma cells were identified in WG, MPA and proliferative glomerulonephritis.

In WG compared with other non-allergic inflammatory nasal conditions, an increase in IgE-plasma cells in the nasal mucosa has been demonstrated (J Piris, personal communication). The serum IgE concentration was not measured.

An increase in mast cells detectable by IgE immunoperoxidase in inflammatory conditions such as WG, MPA and proliferative glomerulonephritis has been demonstrated. The distribution of mast cells in WG differs from the other two. In WG the release of mast-cell products may permit immune-complex deposition into vessel walls, with increased permeability, and so initiate the progression to vasculitis.

CHAPTER 3

ANTIBODIES TO NEUTROPHIL CYTOPLASMIC ANTIGENS IN WEGENER'S GRANULOMATOSIS AND OTHER CONDITIONS

3.1 INTRODUCTION

3.1.1 Antibodies to Neutrophil Cytoplasm Antigens

Most laboratory findings in WG are non-specific and only confirm the presence of systemic illness. Serum complement levels are usually normal (Howell and Epstein, 1976; Pinching et al, 1983; Ronco et al, 1984) although some patients have an increased turnover of complement components (Roback et al, 1969). Mild anaemia, moderate peripheral blood neutrophilia and thrombocytosis are common (Fauci et al, 1983). Although hyper-gammaaglobulinaemia is common (De Remee et al, 1976; Howell and Epstein, 1976; Conn, 1976; Appel et al, 1981) significant autoantibodies are rare. Rheumatoid factor may be present in low titres, but have no diagnostic value (Howell and Epstein, 1976; Ronco et al, 1984; Noritake et al, 1987; Brenner and Lezarrus, 1988). Both the erythrocyte sedimentation rate and C-reactive protein concentration are raised (Fauci et al, 1983; Hird et al, 1984) and CRP is one of the best markers available for monitoring disease activity (Ludemann and Gross, 1987).

In 1985, Van der Woude and colleagues described the presence of IgG antibodies in the serum of patients with active WG which were directed against some cytoplasmic component of neutrophils from healthy volunteers. They found no evidence of antibodies to neutrophil cytoplasmic

antigens (ANCA) in any other condition or in normal controls. The ANCA they described gave a coarse, cytoplasmic fluorescence in an indirect immunofluorescence test where patient serum was incubated with cytospin preparations of neutrophils. Furthermore their preliminary studies indicated that ANCA titres fell during clinical remission of disease activity. They did not find, however a very close or consistent relationship between antibody titre and disease activity. There was therefore a need to evaluate ANCA in diagnosis of WG and in monitoring of disease activity and to see if the differences existed in the pattern of fluorescence obtained with these newly discovered autoantibodies indicating that a variety of different antigens may be recognised (Sayers et al, 1979). Furthermore it was of interest to inquire whether other diseases with a vasculitic component and known immune disturbance (eg IgA nephropathy and Henoch-Schonlein purpura) had ANCA of other immunoglobulin class than IgG.

3.1.2 Enzyme Linked Immunosorbent Assay for ANCA

Immunofluorescence assay for ANCA is rapid, simple and cheap (Van der Woude et al, 1985), and an estimate of antibody concentration can be made by serial dilution of serum samples tested. Since separation of cells for immunofluorescence and fixation of cytospins may introduce

variations reducing the reliability and reproducibility of immunofluorescence assays (Dighiero et al, 1987) enzyme linked immunosorbent assay (ELISA) has become a generally accepted method for detection and quantification of antibodies (Wheeler et al, 1988). In ELISA the specificity depends on the preparation of antigen and its purity, and the sensitivity is determined by the solid phase used, usually plastic microtitre plates. The background reactivity (Tijssen, 1985) and interassay variations result from artefactual effects of the plastic forming the plates (Stemshorn et al, 1983). In work described in this chapter an ELISA method was developed to detect ANCA using microtitre plates coated with whole neutrophils. The initial preparatory stages involved conversion of ANCA assay on cytospins from an immunofluorscence to an immunoperoxidase method.

3.1.3 Target antigens of ANCA

It is clear that IgG ANCA, at least as defined by indirect immunofluorescence, are a heterogeneous group of autoantibodies (Rasmussen et al, 1988). However it is also evident that coarse, granular, cytoplasmic fluorescence is highly specific for WG. Since patterns of fluorescence may not always be reliable and reproducible (Dighiero et al, 1987) it would be of interest to further characterise ANCA by identifying the target antibodies in

the neutrophil cytoplasm. If ANCA from different patients have the same antigen-specificity then this implies a specific antigen-disease association of possible pathogenetic significance (Roitt and Cooke, 1987; Wick et al, 1987). If the antigen specificity of ANCA is different between patients then this may help to explain the different clinicopathological manifestations, in a way similar to that occurring in autoimmune myocarditis (Schultheiss and Bolte, 1985; Schultheiss and Schwimmbeck, 1986) and Grave's Disease (Kohn et al; 1986).

This study sought to characterise further the component(s) of neutrophil cytoplasm against which ANCA are directed by separating proteins according to solubility and molecular weight prior to Western Blotting. It also investigated whether "non-Wegener's" ANCA positive sera have the same specificity to extracted neutrophil proteins.

3.2 MATERIALS AND METHODS

3.2.1 Detection of ANCA by Immunofluorescence

Serum Collection

Clotted blood (5mls) was allowed to separate for 1 hour at room temperature. Serum was obtained by centrifugation and aliquots were stored at -20^{o} until use.

Separation of Neutrophil Polymorphonuclear Granulocytes from Whole Blood

Neutrophil polymorphonuclear granulocytes were isolated using a modification of the method described by Segal and Peters (1977). 3mls of fresh venous blood was withdrawn from healthy volunteers. 15ml aliquots were mixed with 2ml of 2% EDTA in Dulbecco's modified Phosphate Buffered Saline (PBS) (Flow Labs (UK) Ltd) in a plastic universal container (Sterling). 7.5ml of 5% Dextran (MW 250,000D) in PBS was added to each universal container and erythrocytes were allowed to sediment for 45 minutes at 37^{o}C. The supernatant, containing a leucocyte enriched suspension was pipetted off and made up to a volume of 25mls with 10 mM EDTA in PBS. This was centrifuged at 800 rpm for 10 minutes at 4^{o}C (Mistral, Fisons). The supernatant was discarded and the pellet washed once in 25ml 10mM EDTA in PBS.

The pellet obtained was then resuspended in 5ml of 10mM

EDTA in PBS, on to 10ml of Ficoll/Hypaque (Pharmacia, UK) and centrifuged at 1400 rpm for 20 minutes at 4°C.

Under these conditions granulocytes collect below the Ficoll/Hypaque cushion, whereas lymphocytes and monocytes are retarded. The granulocyte pellet was resuspended in 15ml of hypertonic buffer (0.155M NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA) for 15 minutes to lyse contaminating erythrocytes. Neutrophils were collected by centrifugation at 800 rpm for 10 minutes at 4°C, and washed twice in 10mM EDTA in PBS.

The granulocytes were washed in 10ml of PBS containing 10mM ß-mercaptoethanol (BDH,UK) 4 ug dithiothreitol (DTT) (Sigma, UK), and 125 ug phenylmethylsulphonyl fluoride (PMSF) (Sigma, UK). The DTT and PMSF were added to the solution just prior to use. The final pellet was obtained by centrifugation at 800 rpm for 10 minutes at 4°C (Mistral, Fisons).

Its composition was confirmed by preparing smears of the final solution, fixed for 5 minutes in ethanol and stained with Giemsa.

Immunofluorescence Technique to Demonstrate IgG ANCA

The technique used was an adaption of the method of Van der Woude et al (1985).

Cytospin slides were prepared from a granulocyte cell

suspension containing 50 x 10^4 neutrophils per ml PBS containing 10mM EDTA using Shandon Cytospin 2 (Shandon Southern Products, UK). Slides were placed in the cytospin and a 100 ul of the granulocyte suspension was added to each bucket and spun at 400 rpm for 4 minutes.

The slides were air dried for 30 seconds and fixed in absolute ethanol, at 4°C for 5 minutes. The slides were stored at -20°C until use and were kept for up to 30 days.

Test serum was diluted 1:20, 1:80 and 1:160, or until fluorescence decreased, in PBS. 200 ul of each dilution was pipetted onto the slide and incubated for 45 minutes at room temperature. The slides were then washed twice in PBS for 10 minutes. 200 ul of fluorescein isothiocyanate (FITC) conjugated sheep anti-human IgG antibody (SAPU, UK) diluted to 1:50 in PBS, was added and slides incubated for 30 minutes at room temperature; both incubations were in humidified, closed slide chambers. Slides were washed in PBS with 2 changes over 20 minutes, and mounted with glycerol/PBS.

Slides were examined using incident light on Leitz Ortholux microscope fitted with an HBO 200 lamp and HP and KP filters with a K510 barrier filter. The pattern and intensity of fluorescence was recorded.

Test serum was compared with known positive controls (originally supplied from Van der Woude's laboratory) and

known negative controls (serum from a normal healthy individual). Slides were also incubated with FITC conjugated sheep anti-human IgG antibody only to detect non-specific binding.

The presence of ANCA of Ig class other than IgG was sought in a number of cases using fluorescein-conjugated rabbit polyclonal anti-human IgA, IgM (SAPU, UK) and IgE (Dako Ltd, UK) in an assay similar to that described above.

Patient Diagnosis

For the purposes of assessment of the diagnostic usefulness of the test, patients' clinical diagnoses were supplied without knowledge of the ANCA status, based on clinical history, examination, laboratory and histopathological findings, and response to treatment. Whilst this may have led to inaccuracies it was thought preferable to reassessing cases in the light of the ANCA findings.

3.2.2 ELISA

Immunoperoxidase detection of ANCA on cytospins of neutrophils: (Optimised method).

Endogenous peroxidase was blocked with 1% hydrogen peroxide in methanol for 10 minutes. After rinsing in freshly made ELISA Buffer serum, diluted 1:100 in the same buffer, was added and incubated for 30 minutes at room temperature.

After 2 further washes with ELISA buffer 1:100 horse radish peroxidase (HRP) -conjugated rabbit anti-human IgG (Dako, UK) was added for 30 minutes. Cytospins were then washed in Tris/HCl pH7.6 for 15 minutes and peroxidase activity was demonstrated using a freshly prepared solution of 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma, UK) as a substrate. After couunter staining with haematoxylin slides were mounted and examined.

ELISA Buffer:	Stock solution	500	ml
	NaCl	73	g
	Tween 20	25	ml
	Gelatin	6.25	g
	Distilled water	2000	ml
	pH 7.4 (1M NaOH)		
Stock Solution:	$NaH_2PO4._2O$	5	g
	Na_2HPO_4 (Anhydrous)	2.85	g
	Distilled water	1000	ml
Tris/HCl pH7.7	NaCl	8.1	g
	Tris	0.6	g
	IN HCl	3.8	ml
	Distilled water	1000	ml

DAB Solution	3.3[1] DAB	5	mg
	0.2M Tris/HCl pH7.6	10	ml
	1% H_2 O_2	0.1	ml

Enzyme linked immunosorbent assay: (optimised method)

A neutrophil suspension prepared as before was adjusted to give a cell concentration of 0.5 : 10^6 cells/ml in PBS/10mM EDTA. To each of the 60 inner wells of a 96 well flat-bottomed polystyrene microtitre plate (Dynatech M-129, UK) 100 ul (10^4 cells) of cell suspension was added. After 3 hours at room temperature the plates were centrifuged at 400 rpm for 5 minutes in a Centor 2 (MSE) centrifuge fitted with a two-plate rotor. The supernatant was pipetted off using an eight channel Titertek pipette (Flow, UK) and plates were dried at 37°C for 30 minutes. Cells were then fixed by adding 200 ul of absolute alcohol at 4°C for 5 minutes. After drying plates were used immediately or sealed and stored at -70°C for up to 1 week.

Endogenous peroxidase activity was blocked with 1% hydrogen peroxidase in methanol for 10 minutes. Each well was then washed with ELISA Buffer 10 times over a period of 15 minutes using the Titertek Manual Eight Channel Washer. Test serum was diluted 1:100 in ELISA Buffer and 100 ul was added to each of three wells and incubated at room temperature for 30 minutes. Each sample was studied in

triplicate in randomly selected wells. After a further 10 changes of ELISA buffer over 15 minutes 100 ul of HRP conjugated rabbit anti-human IgG diluted 1:800 in ELISA buffer was added and incubated for 30 minutes at room temperature. After washing with ELISA buffer peroxidase activity was demonstrated by adding 100 ul of freshly prepared solution of o-phenylendiamine (OPD) (BDH Ltd, UK) and incubating in the dark for 25 minutes at room temperature. The reaction was stopped by adding 150 ul of 4 M sulphuric acid.

<u>OPD Solution</u>:	Buffer Na_2HPO_4	22.5	g
	Citric acid	5.6	g
	Distilled water	1000	ml
	Buffer	50	ml
	OPD	20	mg
	30% H_2O_2	10	ul

Wells were colorimetrically read using a CLS 962 Microtitre Plate Photomoter with a 492nm filter (Cambridge Life Sciences, UK).

The colorimeter was zeroed on the average absorbance in six wells not coated with neutrophils but which underwent washes with ELISA Buffer, incubation with HRP conjugated antibody, and development of colour with OPD substrate.

3.2.3 Target Antigens of ANCA

Selection of Patients

Sera which showed the bright coarsely granular pattern characteristic of WG on indirect immunofluorescence (IIF) were classified as ANCA positive sera. Nine sera which demonstrated this pattern of fluorescence were studied: seven of these had been diagnosed as WG and two as MPA. Diagnosis was based upon a combination of (i) clinical history and findings (ii) laboratory findings: for example erythrocyte sedimentation rate (ESR), and concentration of C-reactive protein (CRP) (iii) histopathological findings: for example necrotising granulomatous vasculitis in nasal or lung biopsies and focal segmental necrotising glomerulonephritis in renal biopsies, and (iv) response to treatment (ie cyclophosphamide, azathioprine) in some cases.

The sera in the control group demonstrated no ANCA activity on IIF. A total of 10 ANCA negative sera were studied. This group consisted of both normals and patients with other conditions.

Extraction of Neutrophil Proteins

Proteins from neutrophils, obtained as for indirect immunofluorescence, were extracted in three fractions based upon the solubility in different solvents. Water soluble,

urea soluble and sodium dodecyl sulphate (SDS) -soluble fractions were obtained using a modification on the method described by Patek et al, (1986).

Water Soluble Proteins

The neutrophil pellet obtained from 90mls of venous blood (30ml from three different donors) was resuspended in an equal volume of PBS containing 10mM ß-mercaptoethanol and 50mg of DTT and 125ug of PMSF per 10 ml of solution.

The cell pellet was "snap" frozen in liquid nitrogen and then thawed in a water bath at 37°C. This freeze thawing procedure was repeated over 20 minutes after which the water soluble fraction was obtained by centrifugation at 13000rpm for 15 minutes at 4°C (Mistral, Fisons).

The supernatant, containing water soluble proteins, was pipetted off, snap frozen in liquid nitrogen and stored at -70°C until use.

Urea Soluble Fraction

The pellet remaining after extraction of water soluble protein was washed twice in a large volume of PBS containing 10mM ß-mercaptoethanol by resuspending the pellet in the solution and centrifugation at 13000rpm for 15 minutes.

The pellet was then resuspended in 50mM Tris-HC1 (BDH, UK)

pH 7.4 containing 8M urea and incubated for 2 hours at 25°C, after which the solution was diluted to 4M by adding an equal volume of distilled water. The urea soluble proteins were obtained by centrifugation at 13000rpm for 15 minutes at 4°C and the supernatant was pipetted off, snap frozen in liquid nitrogen and stored at -70°C until use.

SDS Soluble Fraction (non water/non urea soluble)

The pellet remaining after urea soluble proteins had been extracted was washed twice in 4M urea. The pellet obtained after centrifugation was resuspended in twice the volume of "solubilising solution" -0.01M Tris-Acetate pH 9.0 containing 1% SDS, 0.001% EDTA and 0.1% ß-mercaptoethanol, and incubated for 3 hours at 37°C.

The opaque viscous liquid resulting was boiled vigorously for 20 minutes, or until the viscosity had reduced. At this stage no insoluble precipitate remained. SDS-soluble proteins were "snap" frozen in liquid nitrogen and stored at -7°C until use.

Protein Quantification

The protein concentration in samples was measured using the Biorad microassaay technique.

Standard solutions containing known concentrations of proteins were prepared using a stock solution containing 1mg/ml Bovine Serum Albumin (BSA) (Sigma, UK). 5ml of 20%

Biorad protein assay dye solution (Biorad, UK) was added, mixed and left for 5 minutes.

The optical density (OD) of the standards was measured using light of 600nm wavelength (Phillips spectrophotometer, Cambridge Instruments Ltd). A graph of optical density versus amount of protein was plotted. The amount of protein present in the samples was calculated from the graph after obtaining the optical density using the neutrophil protein extract solution.

Polyacrylamide Gel Electrophoresis (PAGE) and Staining of Gels for Protein

Proteins were separated on the basis of molecular weight using SDS-polyacrylamide gel electrophoresis (PAGE), (Laemmli, 1970).

Gels were prepared according to the method described by Tijssen,(1987). The resolving gel contained 15% polyacrylamide and the stacking gel contained 5% polyacrylamide.

Neutrophil protein samples were solubilised in electrophoresis sample buffer prior to electrophoresis. For water soluble and urea soluble proteins this buffer was 125mM Tris-HCl pH 7.0 containing 4% SDS, 40% glycerol (BDH, UK), 0.001% EDTA, 10% ß-mercaptoethanol and 0.04% Bromophenol blue (BDH, UK). An equal volume of buffer was

added to the sample and heated for 15 minutes in a water bath at 100°C. The SDS soluble fraction was solubilised in twice the volume of buffer containing 0.01M Tris-Acetate pH 9.0 with 1% SDS, 30% sucrose (BDH, UK), 0.001% EDTA, 0.1% ß-mercaptoethaanol and 0.04% Bromophenol blue and heated for 15 minutes in a water bath at 100°C.

After cooling the samples were centrifuged for 5 minutes at 13000rpm. The supernatant was decanted and stored at -70°C until use.

Each gel lane was loaded with 30 mg of protein. A "Rainbow Marker" containing proteins of known molecular weight conjugated to a dye (Amersham (UK) Ltd) was also run on a separate track.

A non-dissociating buffer system was used (Laemmli, 1970). 2L of electrophoresis buffer (see Appendix B) was added to the stacking gel and separating gel reservoirs, in the LKB vertical slab gel electrophoresis apparatus (LKB Ltd). Electrophoresis was initially carried out at 20mA until the protein front which was visible as a blue band had reached the interface between stacking and resolving gel. The current was then increased to 50mA and the electrophoresis continued until the protein front had reached the bottom of the gel.

Staining of Gels for Protein

Gels were stained for total protein after electrophoresis or after the proteins had been transferred to nitrocellulose filters by soaking in Kenacid blue solution (2g/l Kenacid blue (BDH, UK), 10% glacial acetic acid (BDH, UK), 45% methanol (BDH, UK) for 2 hours. Gels were destained overnight in a 10% glacial acetic acid, 45% methanol solution.

Transfer of Proteins to Nitrocellulose Paper; Molecular Weight Calibration; Staining of Nitrocellulose Paper for Total Protein

Proteins were transferred onto nitrocellulose paper (Hybond C, Amersham (UK) Ltd) using electrophoresis (Bittner et al, 1980). The polyacrylamide gel was washed in transfer buffer (0.01M Tris, 0.1M Glycine, 20% methanol) for 30 minutes.

Transfer electrophoresis was performed at 250mA overnight at 4^{o}C using Biorad Transblot apparatus (Biorad, UK).

Immunodetection

Immunodetection was carried out using a modification of the method described in the Amersham guide (Amersham (UK) Ltd).

1. The nitrocellulose filter was washed in Tris Buffered Saline (TBS) (-25mM Tris, 125mM NaCl) - with three changes over 45 minutes. Non-specific protein binding sites on the nitrocellulose paper were saturated by washing for 3 hours in TBS containing 3% BSA, 2% reconstituted dried milk (Cadbury's UK) and 0.5% Tween 20 with three changes over 45 minutes.

2. The filter was cut into strips which corresponded to the gel lanes and individual strips were incubated with patient serum at the appropriate dilution (1:100, 1:300, 1:500 and 1:1000). Serum was diluted in TBS containing 0.1% Tween 20, 0.25% BSA and 5% goat serum (SAPU, UK). Incubation was for one hour at room temperature. This and subsequent incubations were carried out in sealed plastic bag to reduce the volume of reagent required.

3. The filter was then washed thoroughly in a large volume of TBS containing 0.1% Tween 20 with three changes over 45 minutes, and incubated with biotinylated goat antihuman IgG at a dilution of 1:1000 for one hour, using the same diluent as used for patients serum.

4. After washing thoroughly in TBS containing 0.1% Tween 20 the filter was washed for 15 minutes in buffer 1 (0.1M Tris-HCl pH 7.5, 0.15M NaCl) after which it was incubated with the streptavidin/alkaline phosphatase

complex (Gibco, BRL Ltd) for 15 minutes at room temperature.

5. The filter was washed in a large excess of buffer 1, with two changes over 30 minutes and then washed in buffer 3 (0.1M Tris-HCl pH 9.5, 0.1M NaCl) for 15 minutes.

6. Detection of bound alkaline phosphatase/streptavidin complex was carried out by addition of a substrate for alkaline phosphatase which gave a coloured product. The substrate solution used was bromochloro indolyl phosphate/Nitro blue tetrazolium (BCIP.NBT) (TBS pH 9.5 containing 0.38M BCIP, 0.4M NBT, 10mM MgC_2) (Gibco, BRL Ltd). The reagents and nitrocellulose filter were incubated in a sealed plastic bag.

7. The reaction was stopped by washing filters in a large excess volume of "stop" buffer (20mM Tris-HCl pH 0.5mM Na_2EDTA).

The reaction was allowed to proceed for 3 minutes using the SDS soluble proteins and 5 minutes when the nitrocellulose strips with water soluble and urea soluble proteins were used. After preliminary experiments these were found to be the times where the signal to background ratio was highest.

A peroxidase detection method was also used:

Steps 1-3 were identical to those for the alkaline phosphatase detection method except that the secondary antibody was replaced by peroxidase conjugated goat antihuman IgG antibody (Amersham, (UK) Ltd). The filter was washed in TBS containing 0.1% Tween 20 and 30 minutes and then developed using diamenobenzidine buffer (1.3mM Diaminobenzidine, 0.02% v/v H_2O_2 in 5mM Tris pH 7.4). The reaction was stopped by washing in water.

Controls of Experimental Conditions

Some protein strips were used in different experiments to test the specificity of results. This included (1) detection with omission of serum (2) detection with omission of serum and secondary antibodies (3) detection with only the enzyme substrate.

Analysis of Immunoblots

Blots were analysed by scanning laser densitometry using a chromoscan III (Joyce Loebl) densitometer.

Filters were analysed on a "linear scan" set up on the reflectance mode and using a light source of wavelength 530 mm with a beam size of 0.3 x 0.5mm. The gain (0.15 units) and reflectance (0.1 units) were constant throughout all the analyses and no correction was made for background.

Data was stored on three inch floppy discs (Dysan) and the

incorporated software was used to obtain graphs of optical density versus distance.

3.3 RESULTS

3.3.1 ANCA Immunofluorescence

Epitope Stability

With the combination of filters described background fluorescence was low and there was no difficulty in interpreting results. Although serum stored at -70°C for 4 years retained ANCA activity repeat freeze/thawing on more than 2 occasions led to a marked fall. Cytospins were useable up to 6 months, allowing neutrophil preparations to be made in bulk but in practice Cytospins were always used within 1 month.

IgG Immunofluorescence:

Cytospin preparations were scored according to the nature and brightness of fluorescence giving four groups: a) no significant fluorescence; b) weak, diffuse, cytoplasmic fluorescence; c) bright but not coarsely granular cytoplasmic fluorescence (Figure 3.3.2); d) bright, coarse granular cytoplasmic fluorescence identical to or brighter than, the positive control (Figure 3.3.1). The presence of antinuclear antibodies was also recorded.

No normal volunteer had IgG ANCA activity detected at serum dilutions of 1:20 or greater. Results and clinical diagnoses are summarised in Table 3.3.1. The scoring

system was reliable and reproducible and two other pathologists not involved in the project quickly concurred with the categories already assigned. This was helped by the inclusion of a known positive in each assay.

Sera from 22 patients gave bright, coarsely granular, cytoplasmic fluorescence for IgG. All these patients had systemic vasculitis; 19 had WG, giving a specificity of 86% for this pattern of ANCA activity. In total 26 patients with WG were studied before any treatment. Two of these had evidence only of upper respiratory tract lesions. The sensitivity of the coarse fluorescence pattern for WG was therefore 19/26 or 73%. When the dilution of positive serum was increased the pattern of fluorescence changed from coarsely granular to a less intense diffuse cytoplasmic fluorescence. This made it difficult to accurately record the antibody titre. Two positive sera gave a different pattern of fluorescence at 1:8, most at 1:160 and only one sample continued to give the coarse granular pattern at a dilution of 1:500. For this reason, ANCA were designated as either positive (ie present at serum dilution of at least 1:20) or negative. Comment on the antibody titre was not made routinely. When several patients with coarse, granular ANCA underwent treatment the titre of antibody fell, but more obviously the apparent pattern of fluorescence changed to finely granular or diffuse. Many conditions gave rise to a less intense, more diffuse

cytoplasmic fluorescence which was of no diagnostic value.

Immunofluorescence for Other Classes of Ig

Thirty-five sera were studied including 9 cases of WG and four of IgA nephropathy/Henoch-Schonlein purpura (Table 3.3.2). No serum studied had either IgM or IgE autoantibodies detected, but three cases of IgA nephropathy/Henoch-Schonlein purpura had weak diffuse cytoplasmic fluorescence for IgA (Fig 3.3.3).

TABLE 3.3.1

Summary of the results of immunofluorescence findings for ANCA, according to the nature of cytoplasmic fluorescence and the clinical diagnosis

	Cytoplasmic Fluorescence	No of Cases	Diagnosis
A.	bright coarsely granular	22	WG (19) Microscopic polyarteritis (2) Churg-Strauss (1)
B.	bright	21	WG (2) Microscopic polyarteritis (5) Mixed connective tissue disease (3) Paget's disease of bone (1) Churg-Strauss (1) Bechets Syndrome (1) Wegener's treated (2) Nephrosclerosis (2) Sweet's Syndrome (2) Membranous glomerulonephritis (1) Pulmonary Hypertension (1)
C.	weak	46	WG (2) Microscopic polyarteritis (5) Mixed connective tissue disease (2) Rheumatoid arthritis (3) Churg-Strauss (1) Paget's disease of bone (3) Goodpasture's (1) Sweet's Syndrome (2) Other (27)
D.	insignificant	220	WG (1)+ Microscopic polyarteritis (2) Including: Wegener's limited to the upper respiratory tract (2) Sweet's Syndrome (8) Pneumonia(6) nasal polyps(3) myeloma(3) Bechets syndrome(3) sarcoid(4) Good-pasture's(5) Polymyalgia rheumatica(4) Wegener's treated(11) Polyarteritis treated(8) malignancy(12) other inflammatory conditions(148)

+This case had weak antinuclear antibody activity. Lung biopsy showed a typical granulomatous necrotising vasculitis.

TABLE 3.3.2

Immunofluorescence findings for a variety of conditions investigated for the presence of ANCA immunoglobulin classes other than IgG

	Total Number	IgG (includes granular and diffuse ANCA)+	IgM	IgA	IgE
Wegener's Granulomatosis	9	9	0	0	0
Churg-Strauss	3	3	0	0	0
Microscopic Polyarteritis	4	4	0	0	0
Henoch-Scholein purpura	2	1	0	1*	0
IgA nephropathy	2	1	0	2*	0
Systemic lupus	2	0	0	0	0
Glomerulonephritis	7	1	0	0	0
Normal	10	0	0	0	0
TOTAL	39	19	0	3	0

+ Groups b,c,d, as defined in 3.3.1. (page 89)

*In these cases there was weak, diffuse cytoplasmic fluorescence at serum dilutions up to 1:40 (Figure 3.3.3)

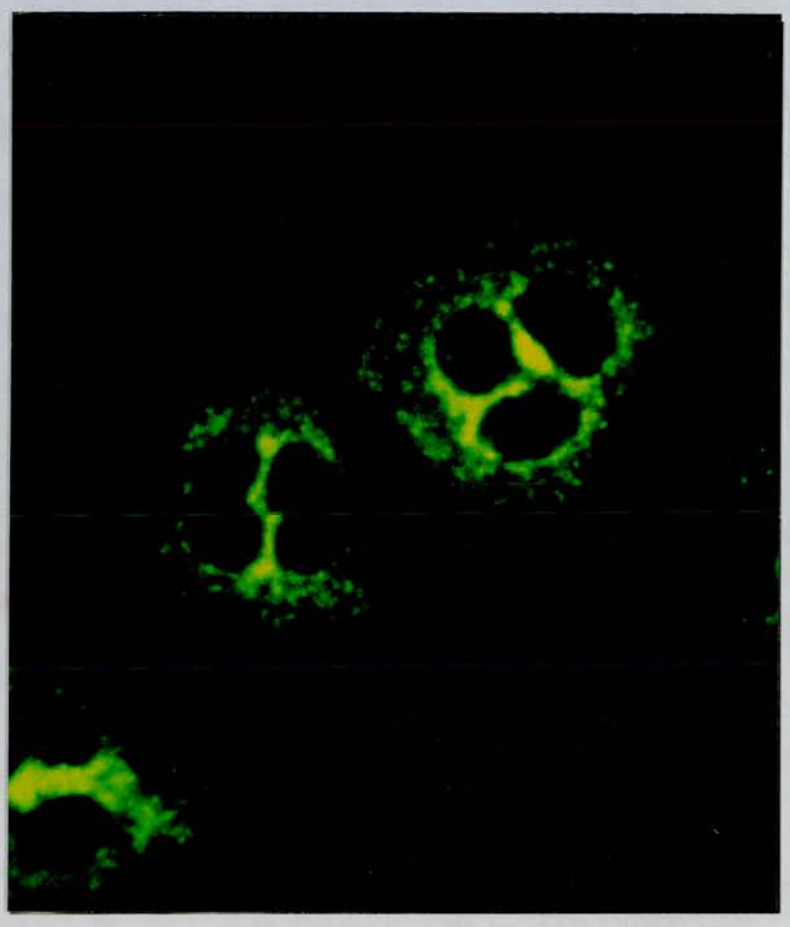


FIGURE 3.3.1 Neutrophil cytospin showing coarse, granular cytoplasmic fluorescence after incubation with WG serum.

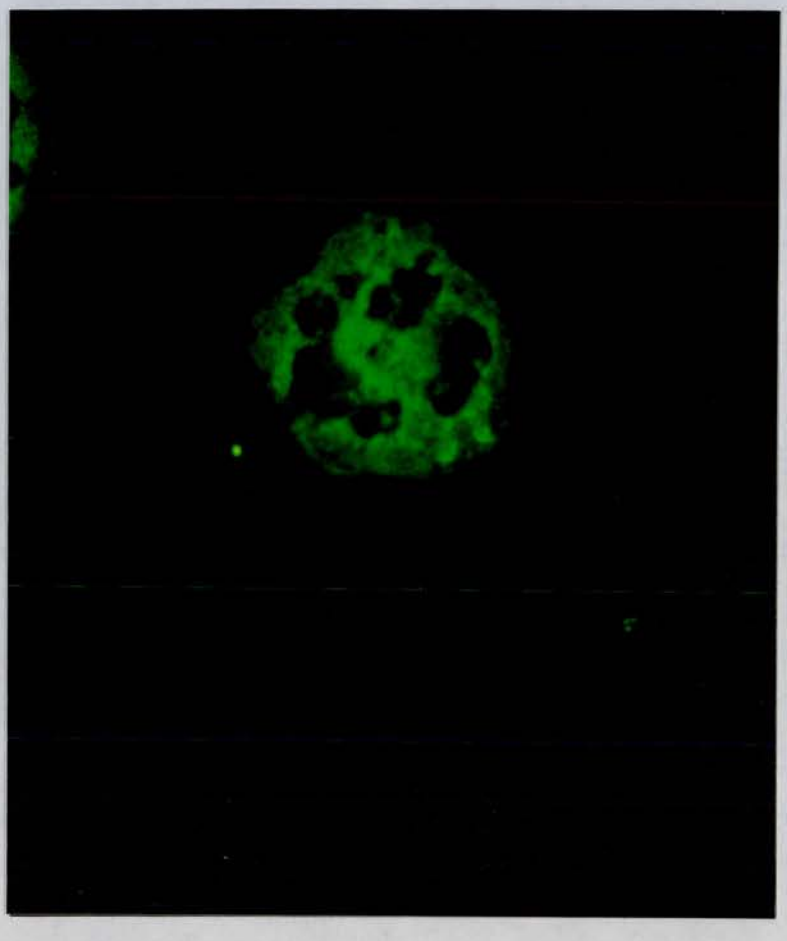


FIGURE 3.3.2 Photomicrograph of neutrophil cytospin showing a more diffuse cytoplasmic fluorescence for IgG after incubation with MPA serum.

FIGURE 3.3.3 Photomicrograph showing weak diffuse cytoplasmic fluorescence for IgA after incubation of cytospins with serum from a patient with Henoch-Schonlein purpura.

3.3.2 ELISA

Standardisation of Technique

The major initial problem was of non-specific binding of both serum and secondary antibody. This was prevented by alteration of the ELISA Buffer to include gelatin in addition to Tween 20. Trypsinisation of the fixed cells had no effect. Because of the possibility of endogenous IgG being bound to Fc receptors on neutrophils giving a false positive result fixed cells were pretreated with Rabbit Fab fragment anti-human IgG. This had no significant effect on light absorption even when used at dilutions of up to 1:2.

The number of cells used to coat plates was determined by experiment (Figure 3.3.4) and 5.10^4 cells was selected. Patient serum at a dilution of 1:100 gave the best discrimination between the test positive and negative sera (Figure 3.3.5). The dilution of HRP-conjugated secondary antibody chosen was that in use in other ELISA systems (P Cachia, Personal Communication). Incubation of sera for longer than 1 hour led to significantly increased non-specific binding giving higher background results.

In order to minimise intra-assay variation only the central 60 wells of the 96 well plate were used to avoid edge-effect (Stemshorn et al, 1983) and replicate samples

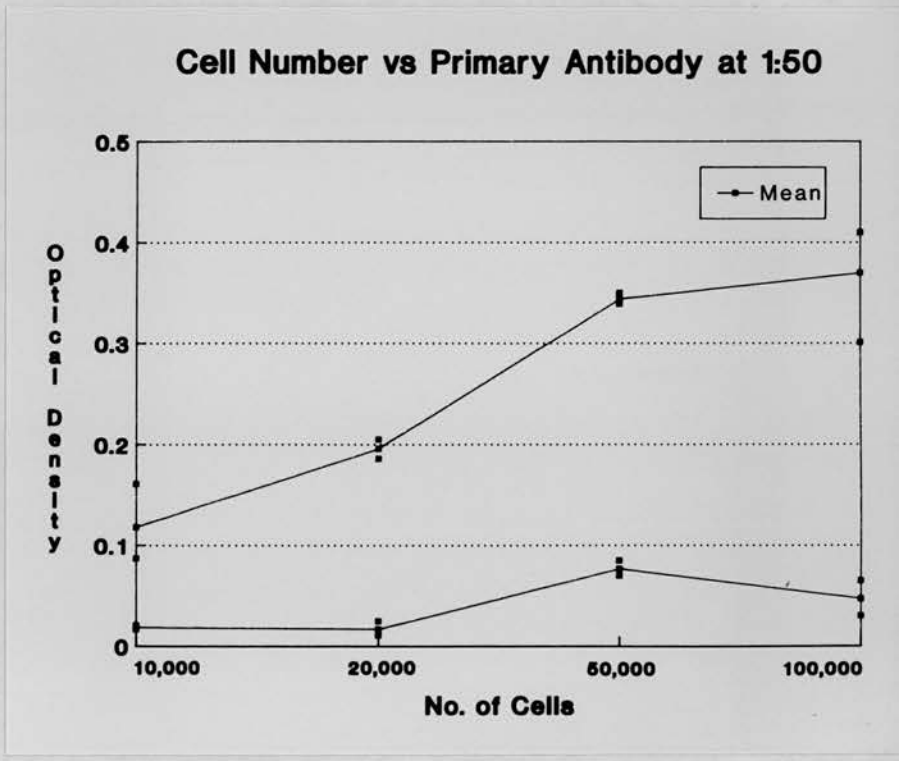
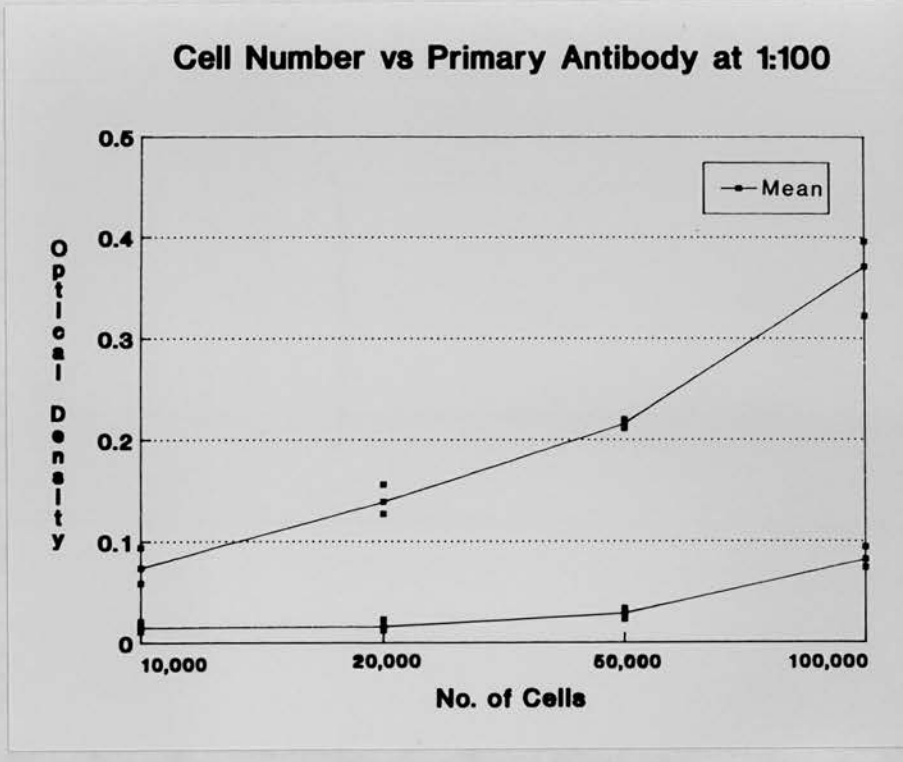


FIGURE 3.3.4 Graphs of optical density against cell number showing assessment of optimal cell number for ELISA plates at a serum dilution of 1:50. For the actual comparison of patient samples $5\text{-}10^4$ cells were needed to coat plates.



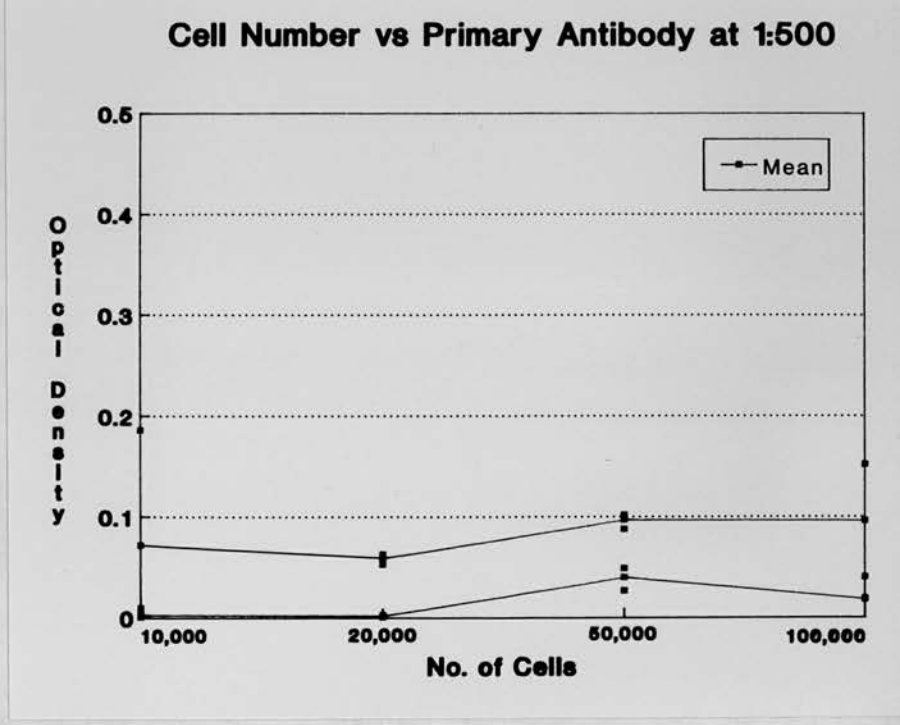


FIGURE 3.3.5 Graphs of optical density against cell number for two dilutions of serum. For comparison of patient samples serum was diluted 1:100 (see also Fig. 3.3.4).

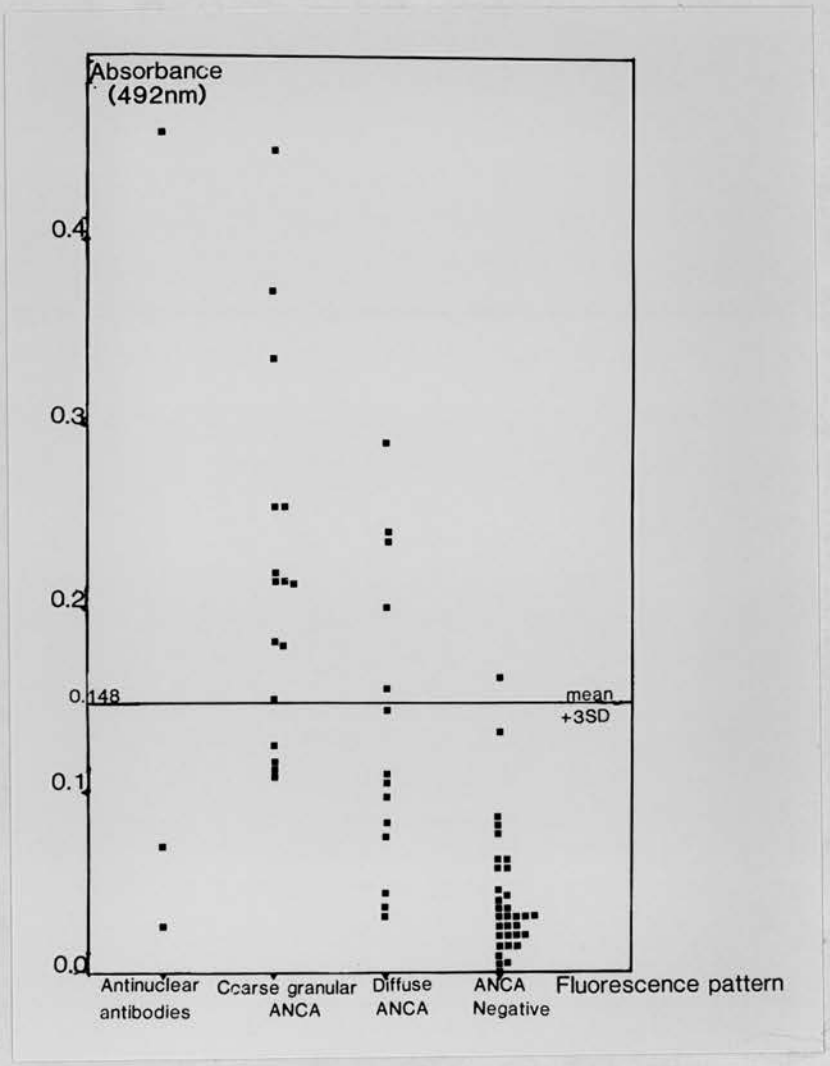


FIGURE 3.3.6 ELISA results (optical density) obtained for each of the patient groups studied. (The horizontal line represents the mean + 3 standard deviations of the ANCA negative control group).
Cases with diffuse or negative ANCA represented a wide variety of diagnoses.

were distributed in randomly selected wells (Wheeler et al,1988).

The coefficient of variation within and between separated assays (ie microtitre plates) was calculated by Tijssen's (1985) modification of Robard's method (1974) (Table 3.3.3).

Using a positive sample the coefficient of variation for means of triplicate samples within assays was 6.6%. The coefficient of variation for means of triplicate samples between assays (ie the variation of reproducibility) was unacceptably high at 29%. The coefficient of variation for negative samples with low absorption readings were lower.

ELISA Results for Patients' Sera

For the preliminary assessment of the usefulness of the ELISA technique 67 different sera were studied. This included patients with coarse granular fluorescence (n=16), finely granular/diffuse fluorescence (n=14) and no ANCA activity detectable (n=33). Results are shown in Figure 3.3.6. Whilst the absorbance values for coarse granular ANCA sera were significantly higher than ANCA negative sera ($p < 0.01$, Mann-Whitney U Test) there was overlap between the two groups. If the upper limit of "normal" is arbitrarily designated as mean (ANCA negative sera) +3

Standard Deviations (Tijssen, 1985) then there was one "false positive" in the ANCA negative group, but there were 4/16 "false negatives" in the coarsely granular ANCA positive group. Furthermore the lack of specificity of the assay using whole neutrophils as the coating antigen means that no distinction could be made between different patterns of fluorescence, whether nuclear or cytoplasmic.

TABLE 3.3.3

The calculation of coefficients of variations for an ANCA positive sample.

ASSAY NUMBER	ABSORPTION 1	2	3	MEAN	STANDARD DEVIATION
1	0.217	0.220	0.211	0.216	0.005
2	0.333	0.211	0.225	0.256	0.067
3	0.168	0.200	0.179	0.182	0.016
4	0.353	0.0389	-	0.371	0.025
5	0.285	0.349	0.370	0.335	0.044
MEAN	0.271	0.274	0.246	0.272^{o}	0.031
STANDARD DEVIATION	0.078	0.085	0.079+		

(i) Coefficient of variation within assays for means of triplicates (%) = $\frac{0.031^{*}}{0.272^{o}} \; 100 \; \frac{1}{3} = 6.58\%$

(ii) Coefficient of variation between assays for means of triplicates (%) = $\frac{0.079+}{0.272^{o}} \; 100 = 29.00\%$

* Standard deviation within assays for single sample (x 1/3 for means of triplicate samples)

\+ Standard deviation between assays for means of triplicate samples

^{o} Mean of means

3.3.3 Target Antigens of ANCA

Clinical

Nine patients with coarsely granular ANCA were studied, 5 of whom were male and 4 female. Their mean age was 49y. (31- 37y). Seven of the ANCA patients had been diagnosed as WG and 2 as MPA.

None of these sera had shown evidence of other autoantibodies on routine screening for anti-DNA antibodies, anti-smooth muscle, anti-mitochondrial antibodies and rheumatoid factor.

Eleven normal controls with no disease and who were ANCA negative were used in this study. There were 6 males and 5 females with a mean age (range) of 39 years (22-70y).

Immunoblotting Results

Protein Extractions, SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Transfer of Proteins to Nitrocellulose Filters

Staining of SDS-PAGE gels showed that the proteins present in the water soluble, urea soluble and SDS soluble fractions were different through the whole range of molecular weights. The pattern of staining was consistent when neutrophils from different donors were used.

Control of Experimental Conditions

Control experiments identified several potential problems.

1. Insufficient blocking of non-specific protein binding sites resulted in the detection of numerous reacting bands at high dilutions of negative sera or using secondary antibody alone. Blocking conditions were altered by increasing the protein content of the blocking buffer and the time of blocking.

2. The biotinylated goat anti-human IgG antibody reacted with some neutrophil antigens (other than endogenous IgG) even when the test serum was omitted. Inclusion of 5% goat serum in the diluent for antibodies solved the problem of the biotinylated antibody binding to neutrophil antigens.

3. There was no evidence of endogenous alkaline phosphatase activity.

4. The peroxidase based immunodetection system gave a very high background under all circumstances although there was no endogenous peroxidase activity. This method was therefore not used.

Experiments Using ANCA Positive and Control Sera

A series of purple bands on the nitrocellulose filter with very little background staining was detected.

Water Soluble Proteins

Nitrocellulose strips containing transferred

electrophoresed water soluble neutrophil protein extract were incubated with sera at dilutions of 1:100, 1:300, 1:500 and 1:1000.

At serum dilutions of 1:100 both ANCA positive and ANCA negative sera gave a large number of bands on immunodetection (Figure 3.3.7). As the dilution of serum was increased the number of bands detected became less, until at 1:1000 there were no significant bands recognised (Figure 3.3.7) At no dilution was any consistent difference detected between ANCA positive and control sera.

One patient with microscopic polyarteritis reacted strongly with a water soluble protein at molecular weight 20-22kD at a serum dilution of 1:100 (Figure 3.3.8). This peak was not present in any other samples studied.

Urea Soluble Proteins

Nitrocellulose strips of urea soluble proteins were incubated with sera at dilutions of 1:100, 1:300, 1:500 and 1:1000.

At low dilutions numerous bands were present using both ANCA positive sera and control sera (Figure 3.3.9). At increasing dilution the number of bands recognised decreased. At a dilution of 1:500 ANCA positive sera were found to react with an antigen of molecular weight 55kD with which control sera did not consistently react (Figure

3.3.10). This was found in 6/9 ANCA positive sera tested and in only 3 of 11 control sera tested.

SDS Soluble Proteins

Nitrocellulose strips of SDS soluble proteins were incubated with sera at dilutions of 1:100, 1:500 and 1:1000. Decreasing the concentration of serum resulted in fewer bands on the nitrocellulose filters following immunodetection.

At serum dilution of 1:500 ANCA positive sera reacted strongly with a protein of molecular weight 27-31kD which control sera did not react with (Fig 3.3.11). All nine positive ANCA sera tested gave a broad band at this molecular weight (Fig 3.3.12). In addition ANCA positive sera also reacted with an antigen of molecular weight 45kD which none of the control sera recognised (Figure 3.3.13) at serum dilution of 1:500.

At serum dilution of 1:1000 some ANCA positive sera still reacted with the 27-31kD antigen, whereas control sera demonstrated no reactivity.The 45kD antigen was not detected.

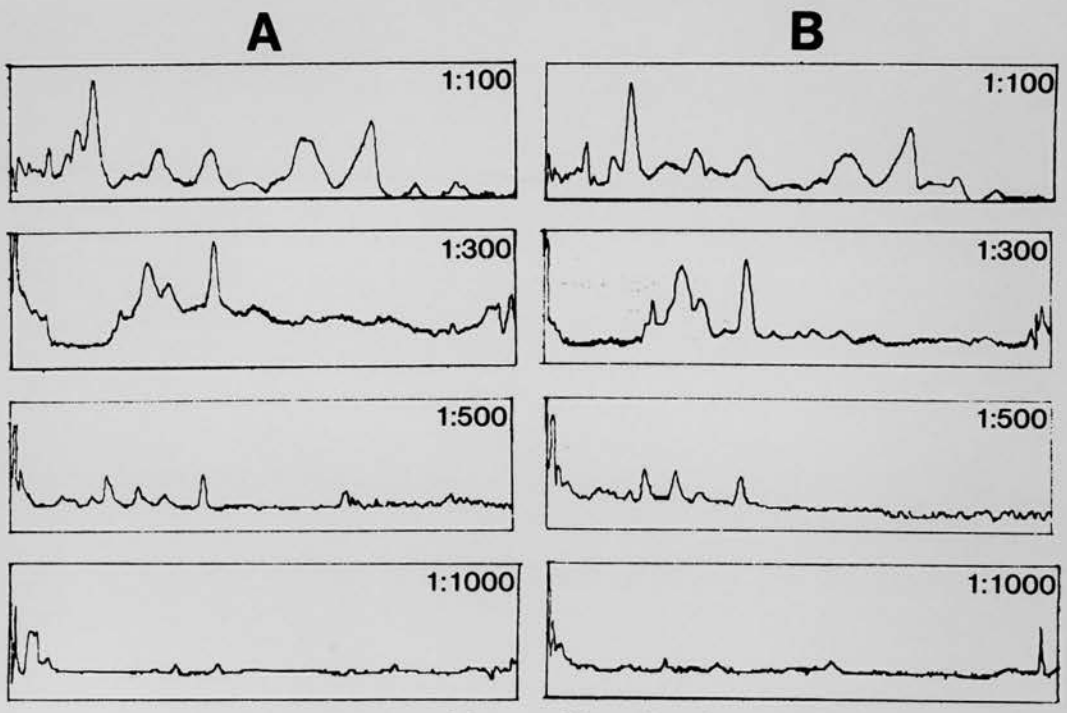


FIGURE 3.3.7 Both ANCA positive and ANCA negative sera recognise many antigens at low dilution of serum but at higher dilutions the number of antigens recognised is reduced. Densitometrograms of decreasing molecular weight versus optimal density.

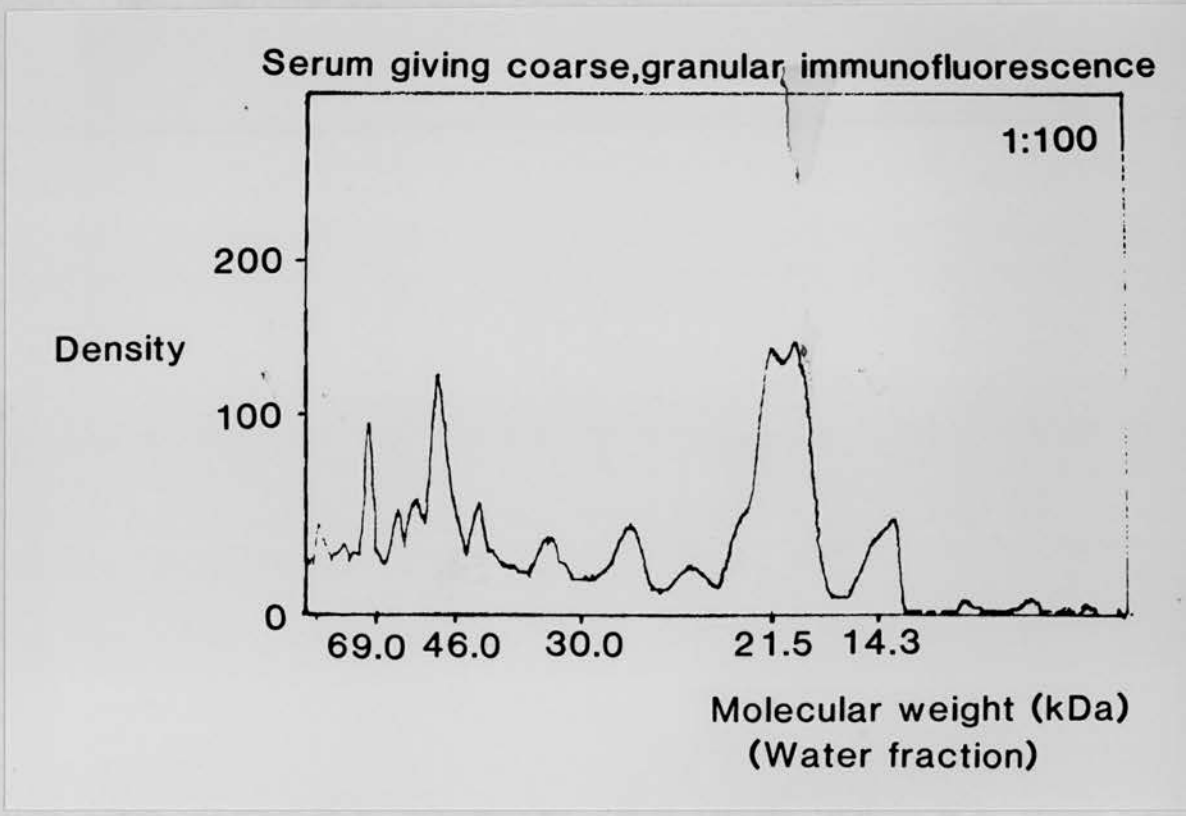
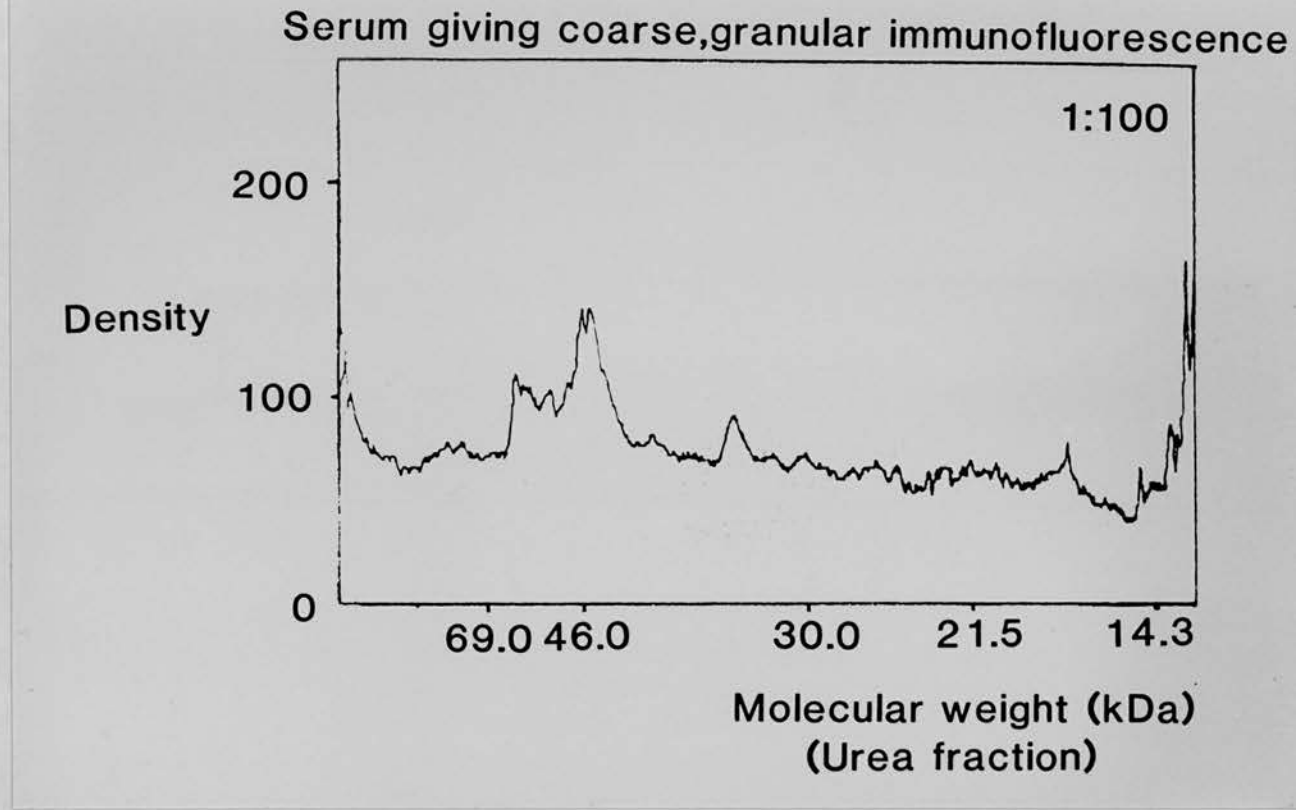


FIGURE 3.3.8 Densitometrogram showing the reaction of serum from a patient with MPA with neutrophil proteins extracted in the aqueous fraction. This serum (1:100) reacted strongly with a 20-22 kDa antigen.



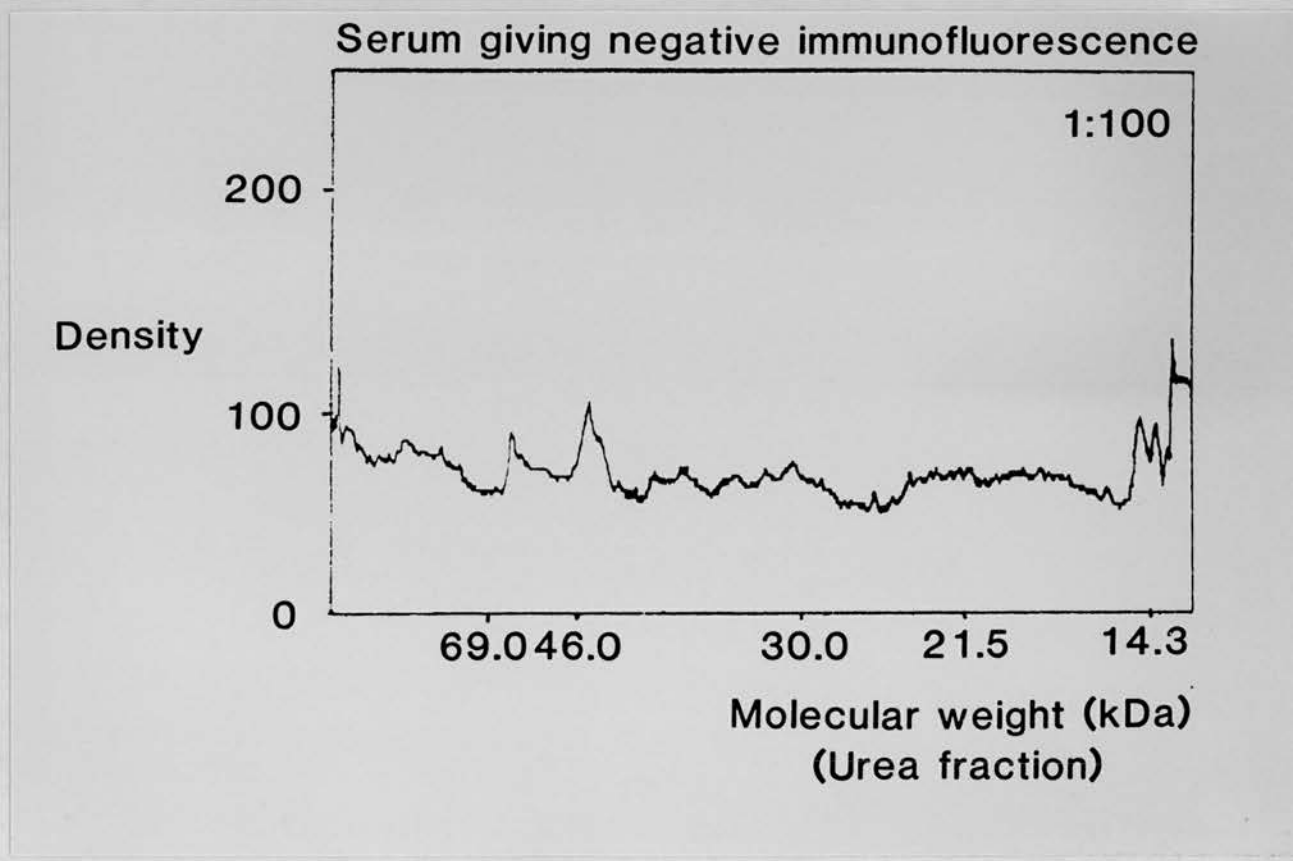


FIGURE 3.3.9 Reaction of ANCA positive and negative sera with urea soluble proteins.

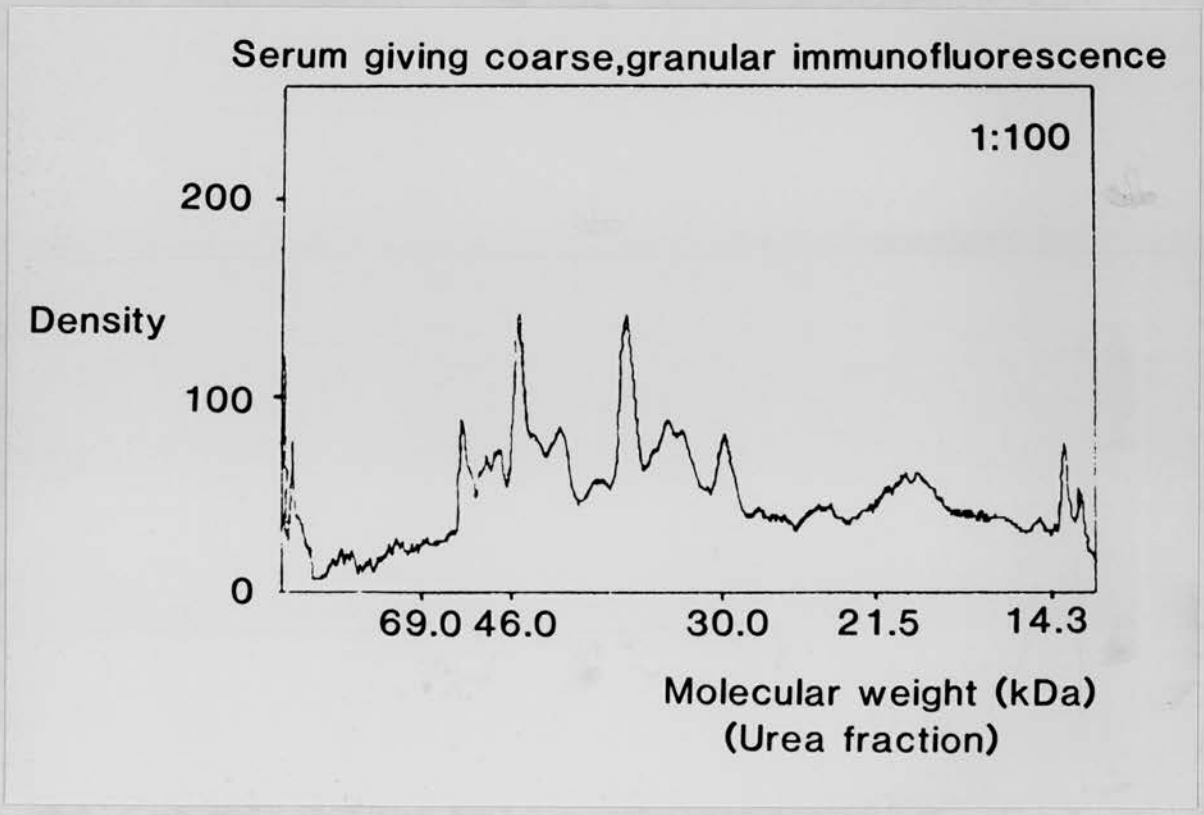
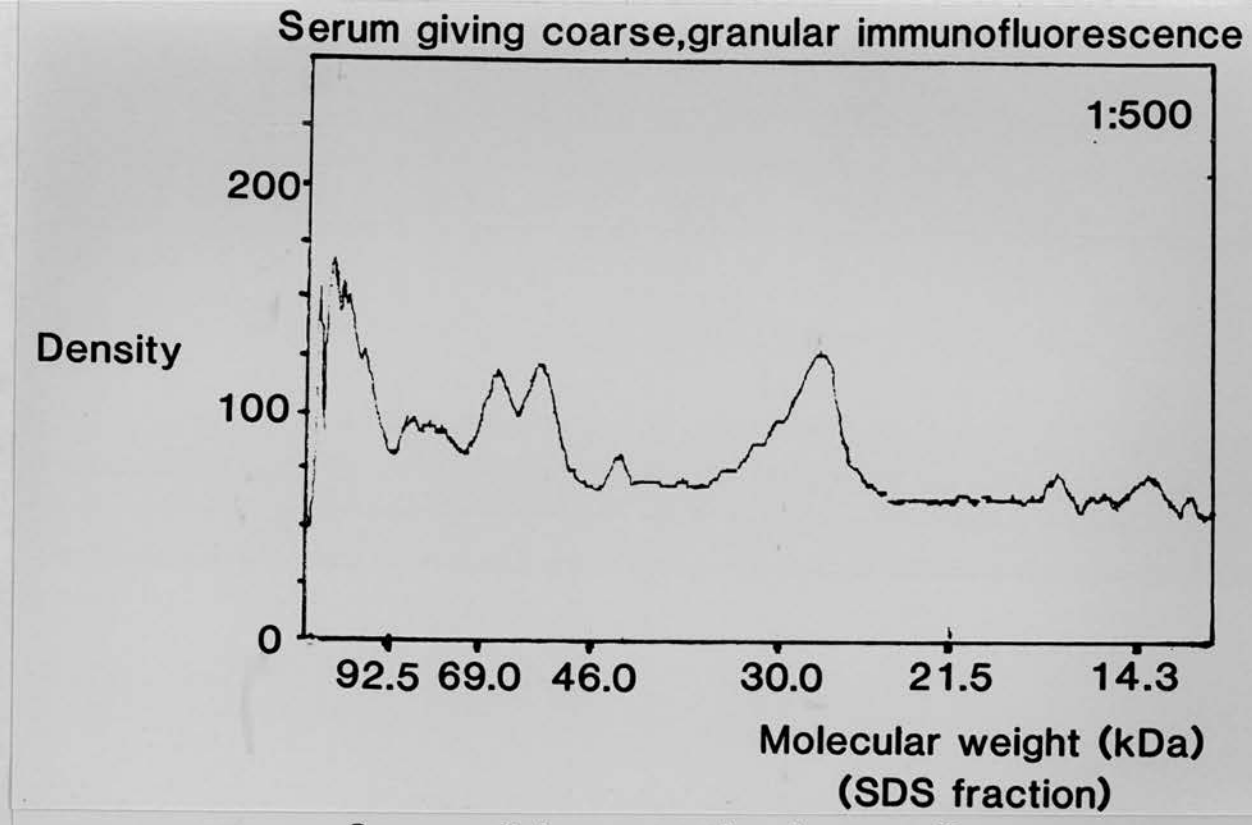


FIGURE 3.3.10 Reaction of ANCA positive serum with a 55 kDa protein in the urea fraction which the ANCA serum does not recognise.



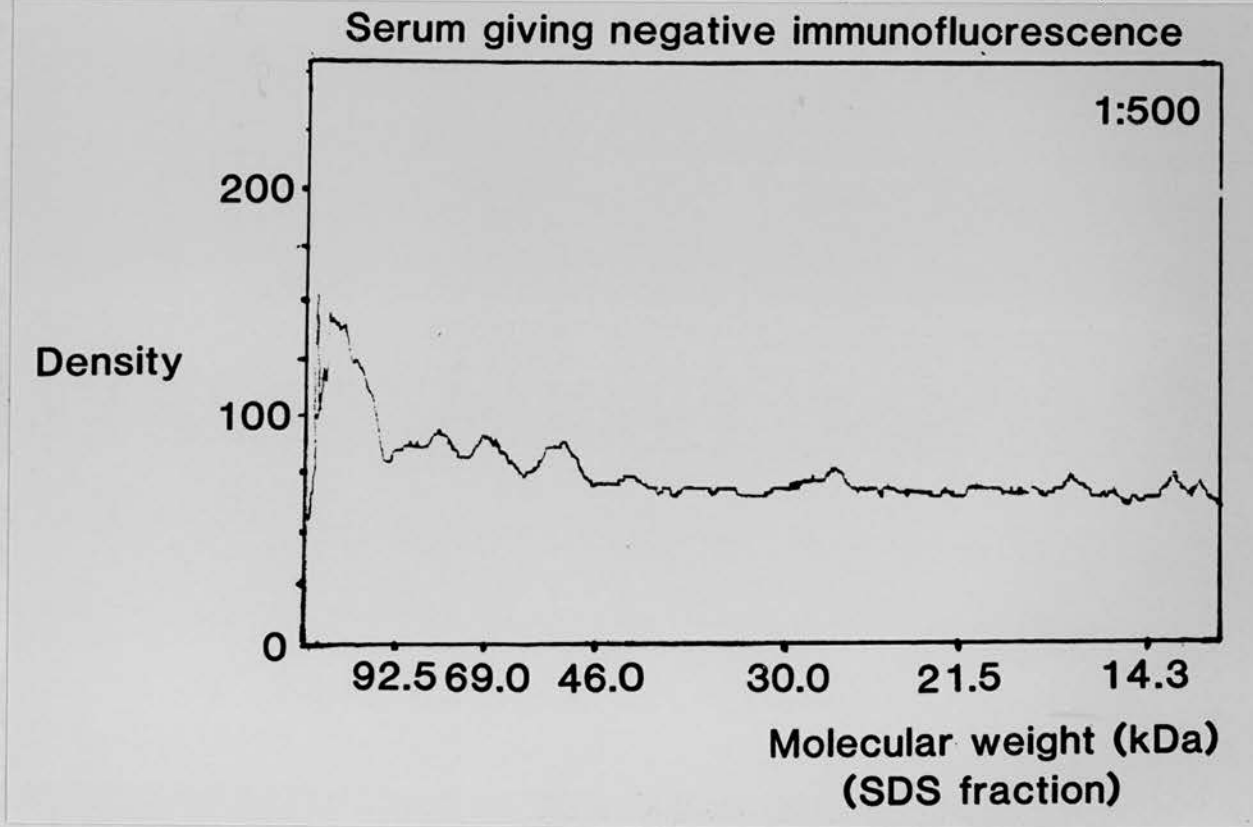


FIGURE 3.3.11 ANCA positive serum recognised a 45kDa and a 27-31 kDa protein in the SDS fraction with which ANCA negative serum did not react.

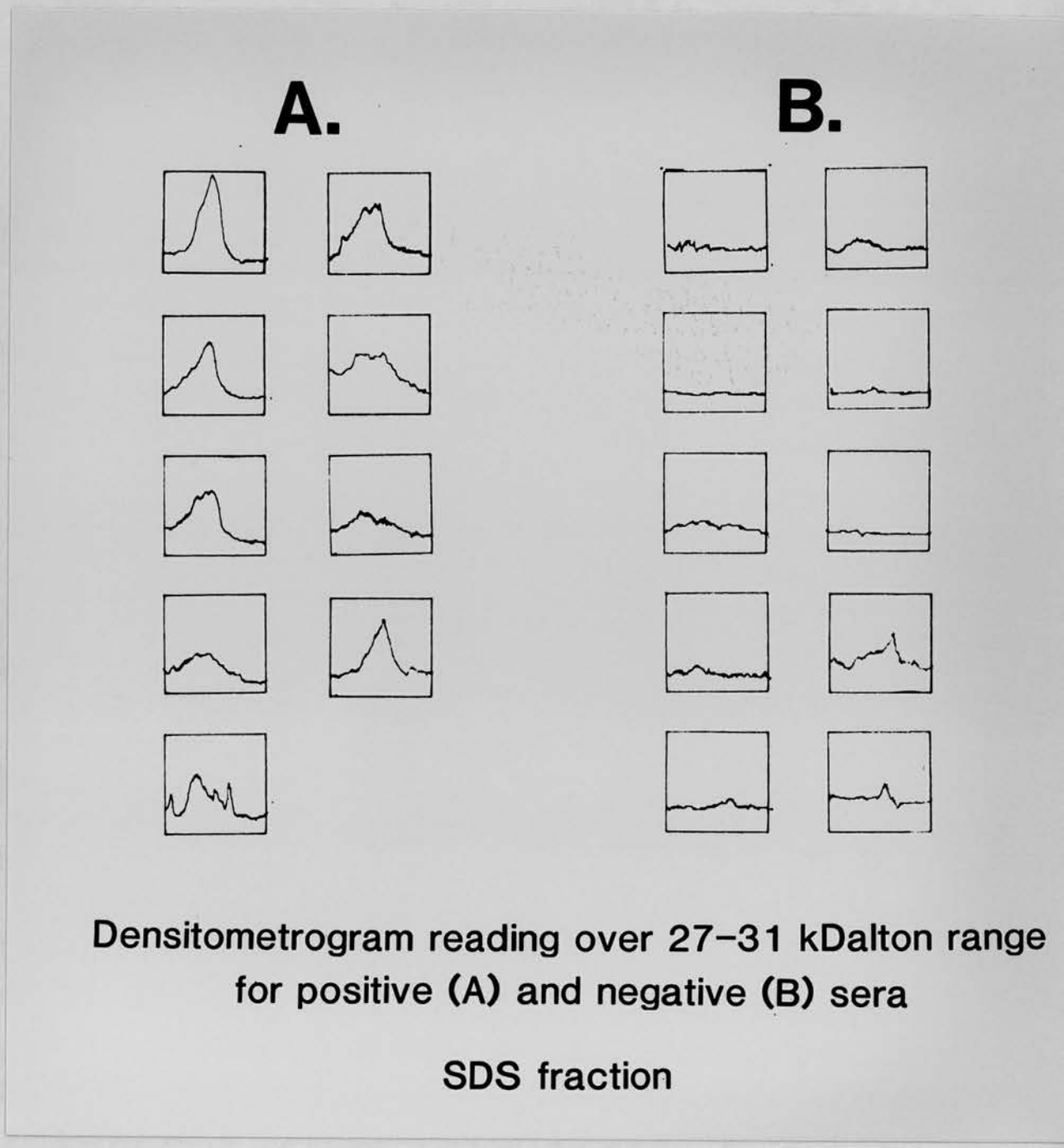


FIGURE 3.3.12 Densitometrograms from positive and negative sera demonstrating that ANCA positive sera recognise a 27-31 kDa protein. (Serum dilution 1:500).

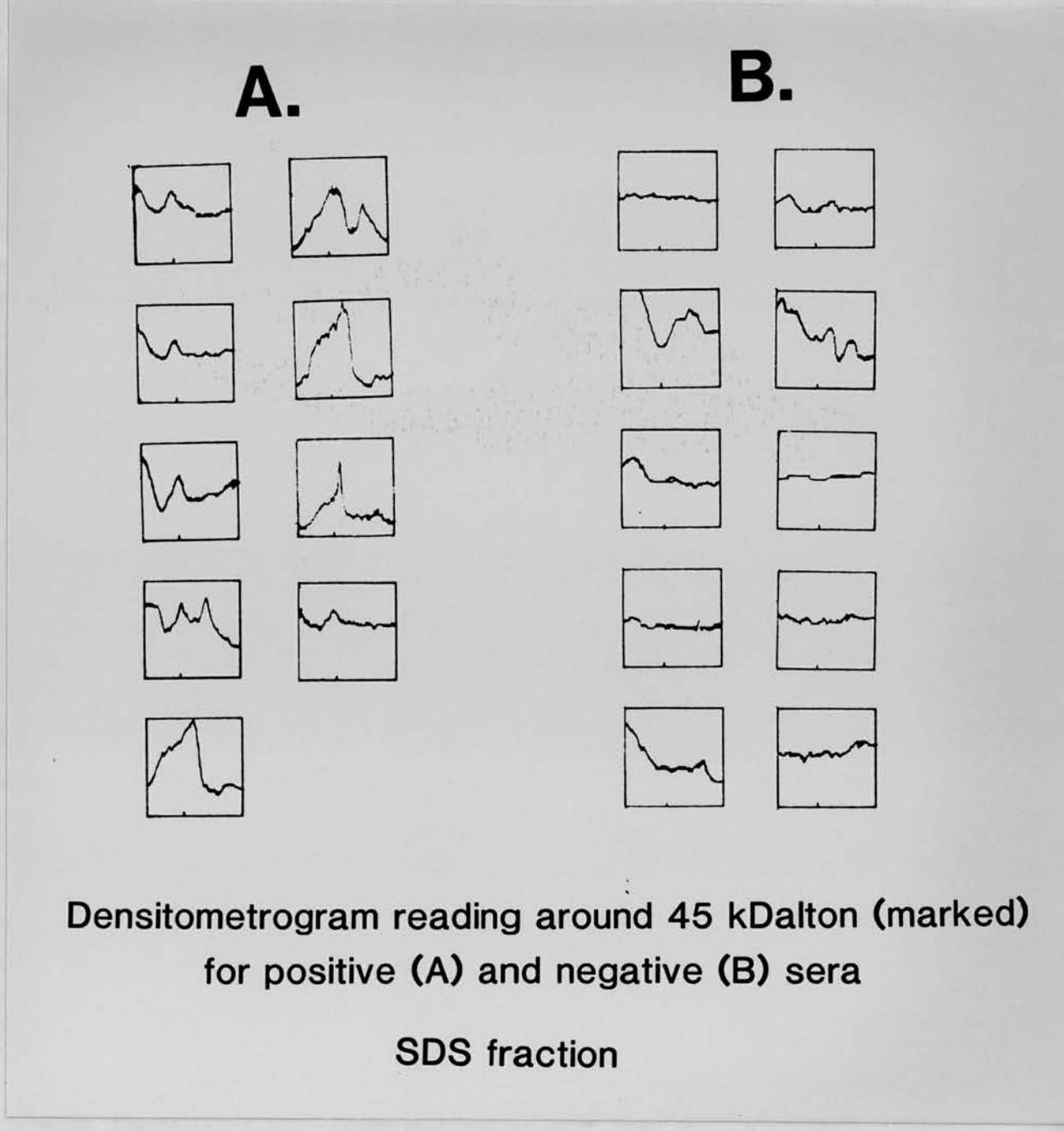


FIGURE 3.3.13 Densitometrograms from positive and negative sera demonstrating that ANCA positive sera recognise aa 45kDa protein (Serum dilution 1:500).

3.4 DISCUSSION

3.4.1 ANCA Detected by Immunofluorescence

The classification of vasculitis is difficult and at times unclear (McCluskey and Fienberg, 1983). It is generally accepted that primary vasculitis is immunologically based, probably involving immune complex mediated damage to the vascular wall (Cupps and Fauci, 1981).

The diagnosis of Wegener's granulomatosis is frequently delayed because of the difficulty in obtaining definitive diagnostic material (Leavitt and Fauci, 1986). The discovery of ANCA, thought to be specific for WG, was therefore hoped to be a significant advance in the recognition of this disease and its differentiation from other vasculitides, in particular MPA (Van der Woude et al, 1985; Ludemann and Gross, 1987).

Although we have found ANCA in cases of MPA and Churg-Strauss Syndrome our specificity of 86% for WG is high, and is comparable to the range of 88-100% reported in other series (Van der Woude et al, 1985; Ludemann and Gross, 1987; Gross et al, 1986; Venning, Arfeen and Bird, 1987; Savage et al, 1987).

The reported sensitivity of ANCA tests for active WG varies from 71% to 100% in the same series which is similar to the 78% sensitivity in our study. These results are

equivalent to accepted values of antidouble stranded DNA antibodies in systemic lupus erythematosus and anti-acetylcholine receptor antibodies in myasthenia gravis (Ludemann and Gross, 1987). In addition to the very bright granular cytoplasmic fluorescence characteristic of WG a large number of samples had ANCA activity, albeit with less intensive and diffuse fluorescence, including 4 cases of WG , 10 of MPA and 2 of Churg-Strauss syndrome. A large number of connective tissue diseases were included in this group as well as several cases of Paget's disease of bone. Diffuse, weak ANCA activity has been described in serum from 4 patients with primary biliary cirrhosis (Gross, Ludemann and Schroder, 1987) 2 cases of rheumatoid arthritis (Savage et al, 1987), one bronchogenic carcinoma and one viral enteritis (Venning et al, 1987). The significance of these findings is unclear but it does suggest that ANCA may be a heterogeneous group of antibodies directed against different epitopic determinants. In cases of mixed connective tissue disease there is a well described antibody to extractable nuclear antigen (Sharpe, 1972). Our present findings of cytoplasmic fluorescence in this condition may be the result of artefactual displacement of nuclear antigen during preparation of neutrophils for cytospins, in a way similar to the proposed displacement of nuclear c-myc oncoprotein during tissue fixation from the nucleus to the

cytoplasm (Williams, Wyllie and Piris, 1987; Loke et al, 1989). The weak ANCA activity found in cases of Paget's disease of bone is of interest. Lockwood and colleagues (1987) have proposed that the target antigen of ANCA in vasculitis is an epitope of alkaline phosphatase, although this is controversial (Gross et al, 1987; Rasmussen, Borregaard and Wiik, 1987; Goldschmeding et al, 1987). If alkaline phosphatase is the autoantigen then ANCA may be an epiphenomenon related to increased serum concentrations of the enzyme as a result of neutrophil degranulation. In Paget's disease the serum concentration of bone alkaline phosphates is raised, and it is possible that ANCA are the result of cross reactivity between epitopes of bone and neutrophil alkaline phosphatase . A similar argument may apply to ANCA activity detected in cases of primary biliary cirrhosis (Gross et al, 1987). In these cases fluorescence is weak and diffuse, similar to that seen using rabbit anti-human alkaline phosphatase antiserum (Rasmussen et al, 1987).

Initial reports of ANCA in WG suggested that the titre of antibody may relate to disease activity (Van der Woude et al, 1985) although the results of prospective trials are not yet published. At present C-reactive protein concentrations are the best serum marker of disease (Ludemann and Gross, 1987) although erythrocyte sedimentation rate may be of additional help (Hird et al,

1984; Fauci et al, 1983). Although the present study found that the titre of antibody decreased with treatment it was difficult to accurately determine antibody titres for several reasons:

1. There was always a low background fluorescence which made the recognition of significant fluorescence difficult at high dilutions.

2. As serum was titrated out the pattern of fluorescence changed from coarsely granular and bright to less intense finely granular or diffuse. This has recently been confirmed by the International Working Group on ANCA (Rasmussen et al, 1988). For these reasons coarse granular ANCA activity was deemed to be positive if it was present at dilutions of at least 1:20, and no attempt was made to use antibody titre, as assessed by immunofluorescence, for monitoring of disease activity.

Many neutrophil granule components or products of neutrophil activation can cause vascular injury (Harlan, 1987). Elastase is present in azurophilic granules and, when released, can detach and lyse endothelial cells in vitro (Harlan et al, 1981; Smedley et al, 1986) and degrade subendothelial matrix, even when bound to alpha-1-antiprotease (Weiss and Regiano, 1984). Elastase released from neutrophils present in serum may therefore be a marker of disease activity. (Wathen et al, 1987). This would be

consistent with widespread, systemic intravascular degranulation of neutrophils in vasculitis, as has been suggested from ultrastructural evidence (Donald et al, 1976).

The finding of weak IgA ANCA in several cases of IgA nephropathy is of interest. This disease is thought to be the result of a disorder in the regulation of IgA responses (Feehally, 1988). Some patients have been described as having IgA autoantibodies which recognise a variety of self antigens and this includes an IgA rheumatoid factor (Sinico et al, 1986). The diffuse nature of the fluorescence would be in keeping with a diverse population of antibodies. The pathogenetic significance of these antibodies is unknown (Feehally, 1988).

In conclusion, IgG ANCA giving bright granular cytoplasmic fluorescence are of great value in the diagnosis of WG and, to a lesser extent, of some other vasculitides. The indirect immunofluorescence assay is rapid, reliable, reproducible, inexpensive and within the capabilities of most laboratories. Further identification of the antigenic epitopes may increase both the specificity and sentsitivity of ANCA in the diagnosis and follow-up of WG, and increase our understanding of the underlying disease process.

3.4.2 ELISA

The sensitivity and specific of any ELISA depends on the antigen preparation, plastic plates used for coating, incubation times, reagent concentrations, freshness of reagents and the degree of difference between positive and negative test sera. The reproducibility of the technique can be determined by calculating coefficients of variation of replicate samples within an assay and between separate assays (Rodbard, 1974). The coefficient of variation between assays should ideally be no more than 3 times the variation within assays, and should be less than 10% (Tijssen, 1985). Clearly the results in this study do not meet these criteria.

The coefficients of variation quoted are calculated from a strongly positive sample. This tends to increase the variation (Wheeler et al, 1988) although in each separate assay the results for that serum would still have been classified as positive. The coefficient for samples giving lower absorbance readings were reduced.

ELISA should also be specific with a clear distinction between positive and negative cases. In our series there is overlap between different groups of sera, and the character of fluorescence is not reflected in the absorbance values obtained. This is because the assay was performed using whole neutrophils rather than purified

antigen. The method is therefore not suitable for qualitative assessment of sera, merely quantitative. Semi purified antigen has been used by other groups (Biocarb, 1988) but still results in some positive results using sera which do not have coarse granular cytoplasmic staining by immunofluorescence. Further identification and purification of the antigens involved is required. Four of 16 sera in this present study with coarse granular cytoplasmic staining gave false negative results, as defined by a cut-off point of the mean of absorbance for ANCA negative sera plus 3 standard deviations. One ANCA negative serum by immunofluorescence gave a false positive result on ELISA. These figures compare unfavourably with other reported ELISA's for ANCA (Biocarb, 1988; Bygren, Rasmussen and Weislander, 1988) where only 2 out of 114 samples gave false negatives. This apparent discrepancy can be explained on the basis of the reference normal ANCA negative population studied. Reported series have used ANCA negative patients' sera. ANCA negative patients' sera may contain increased amounts of reactive proteins and increased concentrations of cross-reacting antibodies which increase the background. Another potential limitation of the present ELISA methodology is its inability to distinguish between the effects of antibody titre in serum and antibody affinity. This may be important since dilution of ANCA positive sera resulted in a changing pattern of fluorescence (affinity and target specificity) before a

loss of fluorescence (titre).

This study has demonstrated that ELISA is of limited usefulness diagnostically, primarily because there is no source of purified antigen at present. With increasing knowledge of the nature of antibodies present in ANCA positive sera and their antigen specificity it may be possible to develop an assay which will specifically identify patients with ANCA of a pattern characteristic of WG, and to sequentially measure antibody titres as a monitor of disease activity.

3.4.3 TARGET ANTIGENS OF ANCA

The presence of "natural autoantibodies" to a variety of self antigens in the sera of normal healthy individuals has been demonstrated previously using competitive enzyme immunoassays and immunocytochemical techniques (Dighiero et al, 1982; Gilbert et al, 1982; Lutz and Wipf, 1982; Holmberg and Coutinho, 1985; Dighiero et al, 1986). Autoantibodies directed against actin, tubulin, thyroglobulin, myoglobulin, albumin, transferrin, collagen, cytochrom C and others have all been demonstrated in the sera of normal individuals, although in lower titres than in pathological states with known antibody association (Dighiero et al, 1986). In addition some human monoclonal antibodies produced by hybridoma techniques using B-lymphocytes from healthy persons or antibodies produced by myelomas have been shown to react, or cross react, with self antigens (Seligmann et al, 1973; Madaio et al, 1986). The recognition of patterns of fluorescence is difficult and there is a need to standardise results between laboratories (Dighiero et al, 1987; Rasmussen et al, 1988).

Autoantibodies associated with specific diseases therefore differ from natural autoantibodies either in the antigen recognised, or the titre of the antibody, or both (Cruse and Lewis, 1985; Kyriatsoulis et al, 1987). These features can be used to distinguish between natural

autoantibodies and disease associated antibodies. However, the identification of disease associated autoantibodies does not necessarily indicate that they are of pathogenetic significance (Graber, 1975; Dighiero, Guilbert and Avrameas, 1982; Cruse and Lewis, 1985; Dighiero et al, 1986; Walport, 1987) (Wick et al, 1987).

Autoantibodies to a variety of nuclear and other antigens are commonly demonstrated in the sera of patients with systemic rheumatic diseases, but their pathogenetic significance remains uncertain (Scott, 1985) Disease associated antibodies are of importance in clinical diagnosis and their serial measurement often provides information on the response to treatment (Cruse and Lewis, 1985; Tan , 1967; Rose et al, 1988), and on occasions autoantibodies have been shown to have a pathogenetic role, particularly in the case of myasthenia gravis (Vincent, 1980; Drachman et al, 1982).

We have confirmed that both control and patient sera contain natural autoantibodies, and have shown that ANCA positive sera recognised epitopes on 45 kDa and 27-31 kDa proteins. Lockwood and colleagues (1987) suggested that the epitopes recognised by ANCA might be part of neutrophil alkaline phosphatase. However their work has been extensively criticised (Gross et al, 1987; Goldschmeding et al, 1987; Rasmussen et al, 1987). They used highly

degraded hydrolysed samples of protein (Goldschmeding et al, 1987) and radioimmunoassay at serum dilutions of only 1:4 which would increase the risk of non-specific binding (Rasmussen et al, 1987). Furthermore, alkaline phosphatase in human, unlike rabbit, neutrophils is present diffusely in the cell membrane and not in cytoplasmic granules (Dewald, Bretz and Baddiolini, 1982) and therefore would not give fine granular immunofluorescence (Rasmussen et al, 1987). The presence of ANCA recognised antigens in the SDS fraction would be consistent with the proteins being membrane associated rather than cytoskeletal (urea-soluble) or cytosolic (water-soluble). Goldschmeding and colleagues (1987) have also reported that ANCA react with a protein of molecular weight 27-29 kDa which they suggest is present in azurophilic (primary) granules. They did not describe a minor antigen at 45 kDa but this may be because they did not perform a differential extract of protein but prepared a whole cell extract. Smaller bands may therefore have been obscured.

The 27-31 kDa antigen gave a broad band on immunodetection and this is consistent with the protein being significantly glycosylated. The broad peak is caused by the analomous migration of glycosylated proteins on SDS-PAGE, probably due to poor binding of SDS (Segrest and Jackson, 1972). This phenomenon has been described

previously in relation to other glycosylated neutrophil proteins (Dinauer et al, 1987). The nature of the 45 KDa antigen is unknown but it is interesting to note that a primary event in the activation of neutrophils is phosphorylation of a membrane associated protein of this molecular weight, and this is defective in some patients with chronic granulomatous disease (Segal et al, 1985; Malech and Gallen, 1987), a condition characterised by granuloma formation. Since SDS denatures proteins it is also possible that the epitopes at 45 kDa and 27-31 kDa are subcomponents of a larger molecule, possibly a dimer. This requires further study using non SDS containing gels.

One serum, from a patient with MPA recognised a water-soluble protein which migrated at the 21 kDa position. The significance of this is unknown but diversity of target antigen specificity has been recognised in other conditions, such as autoimmune myocarditis, where it may determine the pattern of clinical disease (Rose et al, 1988; Schultheiss and Bolte, 1985; Schultheiss and Schwimmbeck, 1986).

It is possible that ANCA in WG and cases of MPA are not directed primarily against the neutrophil, but merely cross-react with it. We did not find any fluorescence when serum was incubated with normal kidney but another group has reported that such cross-reactivity does occur (Abbott et al, 1988).

In conclusion, 2 antigens have been identified which all ANCA positive sera recognised and control sera did not. This finding is of importance, not only in increasing the diagnostic value of ANCA but in obtaining relatively pure antigens for use in improved ELISA and radioimmunoassay techniques to quantify ANCA titres. Further identification of the antigens may increase our understanding of the possible significance of ANCA in the pathogenesis of vasculitis. It is not clear whether the two cases of MPA studied were typical of this condition or whether they were "formes fruste" of WG.

CHAPTER 4

CONCLUSION

4.1 SUMMARY:

Whilst the pathological definition of WG is clear and definite it is apparent that the clinical manifestations may be less easily defined, the latter in part due to the limitations of biopsy sampling, but also the product of the disease itself. WG may present as a "forme fruste", limited disease, or as a systemic disorder involving many organ systems. Clinically overlap syndromes of vasculitis have been described where a disease has features of more than one vasculitic entity. The aetiology and pathogenesis of most individual cases of vasculitis is unknown, although a hypersensitivity reaction to an antigen, such as Hepatitis B virus surface antigen in microscopic polyarteritis, has been implicated in some instances. Such cases have led to the generalised notion that vasculitis is due to immune complex disease; type III hypersensitivity.

In WG, as has been described, the pathognomonic lesion is a granulomatous vasculitis, though granulomata are not a feature of the changes in the kidney. This would be consistent with a cell mediated, type IV, delayed hypersensitivity reaction. However the majority of cases studied had evidence of immune complex deposition in glomeruli and an increase in the number of interstitial and periglomerular mast cells. This prompts the notion that renal (and other?) non-granulomatous manifestations of WG

may be mediated by humoral hypersensitivity mechanisms. Why mast cells in WG and MPA have a different distribution is not clear. It may reflect a different pattern and rate of disease evolution in these conditions. In WG renal disease may follow presentation with respiratory tract lesions whereas in MPA presentation is usually related to renal involvement. There is a possibility that immune complex deposition is merely a reactive or secondary phenomenon occurring in already damaged vessels. However since immune complexes may be found in morphologically and ultrastructurally normal glomeruli this appears unlikely. Neutrophils and their granule contents are thought to be important in the pathogenesis of injury after immune complex deposition. The finding of antibodies with a high specificity for WG which are directed against neutrophil cytoplasm components raises the possibility that WG may be an autoimmune disease. If these antibodies have a pathogenetic role it may be to bind and stabilise released neutrophil granule proteins perhaps reducing neutralisation by serum antiproteases. In a mechanism of this type elastase may play a key role because it can produce vessel wall injury. Elastase has a molecular weight of 29 kDa which is the same as the major protein with which antineutrophil antibodies react. This however remains entirely speculative since no evidence that antineutrophil antibodies have a pathogenetic role has been found. IgG heavy chain isotype restriction would be indirect evidence

of a specific autoimmune disorder but this has not been reported.

In many autoimmune diseases autoantibodies are epiphenomenal and merely serve as markers of disease without having a role in pathogenesis. The antibodies in WG may not be primarily directed against neutrophils but against an exogenous antigen, in a situation analogous to rheumatic fever. Further work is required studying the antigenic components of neutrophils from patients with WG. Alternatively granulomatous inflammation may be indicative of neutrophil dysfunction, as in chronic granulomatous disease of childhood which, perhaps significantly, affects predominantly the upper and lower respiratory tracts. Further functional studies are therefore required.

4.2 THE IMMUNOPATHOGENESIS OF WEGENER'S GRANULOMATOSIS: AN HYPOTHESIS.

Since the lung and upper respiratory tract are almost invariably involved in WG the disease may be related to an inhaled antigen. This results in a delayed type hypersensitivity reaction, possibly with cross-reactivity to host vascular antigens, leading to granulomatous vasculitis. To a variable extent a humoral response is also initiated and this is responsible for most of the systemic manifestations of disease by immune complex deposition. As part of the humoral response antibodies are produced which cross-react with neutrophil proteins. The finding that the antibodies from different patients recognised the same neutrophil cytoplasm antigens suggests that the same initiating exogenous trigger antigen is present in all cases. A lack of significant humoral response would result in disease limited to the upper or lower respiratory tract occurring in association with low titres of antineutrophil antibodies, a combination of findings which is described in this thesis. In other forms of vasculitis such as microscopic polyarteritis, the predominant response to exogenous antigen is humoral. This causes a systematised immune complex vasculitis, and, because many different antigens may be aetiological, there is no consistent cross-reactivity with host antigens. This is consistent with the findings of antineutrophil antibody

heterogeneity found in this study.

The further possibility that there is an inherent or acquired malfunction of neutrophils cannot be excluded.

In many regards the immunopathogenesis of WG remains a mystery, but recent avenues of research are likely to elucidate both environmental and host factors which determine the evolution and course of this disease.

REFERENCES

Abbott F, Jones S, Lockwood CM, Rees AJ.
Autoantibodies to glomerular antigens in Wegener's granulomatosis (WG).
Clinical Science (1988): Suppl 18; 66A

Aldo MA, Benson MD, Comerford FR et al.
Treatment of Wegener's granulomatosis with immunosuppressive agents.
Arch.Intern.Med. (1970): 126; 298-305

Ambrus JL, Fauci AS.
Diffuse histiocytic lymphoma in a patient treated with cyclophosphamide for Wegener's granulomatosis.
Am.J.Med. (1984): 76; 745-747

Appel GB, Gee B, Kashgarian M, Kayslett JP.
Wegener's Granulomatosis - Clinical-pathologic correlations and long-term course.
Am.J.Kid.Dis. (1981): 1; 27-37

Atkins R, Glasgow EF, Holdsworth SR, & Matthews FE.
The macrophage in human rapidly progressive glomerulonephritis.
Lancet i; 830-832

Brenner BM, Lezarrus JM (ed).
In: Acute renal failure. Churchill Livingstone, Edinburgh (1988): 410-414.

Balazs T & Roepke RR.
Lysozymuria induced in rats by nephrotoxic agents.
Proceedings of the Society of Experimental Biology and Medicine (New York) (1966): 123; 380-385

Baker JE, Antonovych T, Fauci AS et al.
The nephritis of Wegener's granulomatosis.
Kidney Intern. (1978): 14; 706 -

Berge IJ, Wilmink JM, Meyer CJ et al.
Clinical and immunological follow-up of patients with severe renal disease in Wegener's granulomatosis.
Am.J.Nephrol. (1985): 5; 21-29

Berman DA, Rydell RE, Eichenholtz A.
Wegener's granulomatosis. A clinicopathological study of 4 cases.
Ann.Intern.Med. (1963): 39; 521-530

Biocarb Diagnostics: Assays for Nephrology. Anti-neutrophil cytoplasm antibodies rapid kit: enzyme immunoassay of ANCA in serum for research on systemic vasculitis. Instruction for use 1988 cat. no. ANC-01.

Bittner M, Kupferer P, Morris CF.
Electrophoretic transfer of proteins and nucleic acids from slab gels to diazobenzylomethyl cellulose or nitro-cellulose sheets.
Anal.Biochem. (1980): 102; 459-471

Bohman SO, Olsen S & Peterson VP.
Glomerular ultrastructure in extracapillary glomerulonephritis.
Acta.Pathologica.Microbiologica.Scandanavia.Section.A (1974): 82; (suppl 249) 29-54

Brentjens JR, Milgorm ML, Andres GA.
Classification and Immunopathologic Features of Human Nephritis. pp 214-254 In: Immunologic Mechanisms of Renal Disease (Ed Wilson CB, Brenner BM, Stein JH) Churchill, Livingstone, New York (1979).

Bygren P, Rasmussen N, Wieslander J.
ANCA in glomerulonephritis.
1st International Workshop on ANCA, Copenhagen 1988 (abstract)

Caralis PV, Falanya V, Pardo V.
Delayed form of Wegener's granulomatosis.
S.Med.J. (1981): 74; 882-883

Cattell V & Jamieson SW.
The origin of glomerular crescents in experimental nephrotoxic serum nephritis in the rabbit.
Laboratory Investigation (1978): 39; 584-590

Cattell V & Aldridge S.
The origin of proliferating cells in the glomerulus and Bowman's capsule in nephrotoxic serum nephritis: Effects of unilateral irradiation.
British Journal of Experimental Pathology (1981): 62; 669-675

Churg J, Grishman E.
Ultrastructure of immune deposits in renal glomeruli.
Ann.Intern.Med. (1972): 76; 479-486

Cochrane CG, Weigle WD, Dixon FJ.
The role of polymorphonuclear leucocytes in the initiation and cessation of the Arthus vasculitis.
J.Exper.Med. (1959): 100; 481-494

Colburn KK, Cao JD, Krick EH.
Hodgkin's lymphoma in a patient treated for Wegener's granulomatosis with cyclophosphamide and azathioprine.
J.Rheumatol. (1985): 12; 599-602

Colvin RB, Dvorak AM, Dvorak HF.
Mast cells in the cortical tubular epithelium and interstitium in human renal disease.
Human Pathol. (1974): 5; 315-326

Conn DL, McDuffie FC, Holley KE, Shroeter AL.
Immunologic mechanisms in systemic vasculitis.
Mayo Clinic Proc. (1976): 51; 511-518

Copeman PWM, Ryan TJ.
The problems of classification of cutaneous angiitis with reference to histopathology and pathogenesis.
Br.J.Dermatol. (1970): 82 (suppl 5); 2-13

Coward RA, Hamdy NAT, Shortland JS, Brown CB.
Renal Micropolyarteritis: A Treatable Condition.
Nephrol.Dial.Transpl. (1986): 1; 31-37

Crofton J, Douglas AC.
In: Respiratory Diseases (3rd Edition).
Blackwell Scientific Publications, Edinburgh (1981): pp 532-540

Cruse JM, Lewis RE.
Contemporary concepts of autoimmunity.
Concepts Immunopathol. (1985): 1; 1-31

Cupps TR, Fauci AS.
In: The Vasculitides.
WB Saunders Philadelphia (1981): 72-87

Dalchau R, Kirkley J & Fabre JW.
Monoclonal antibodies to a human leucocyte-specific membrane glycoprotein probably homologous to the leucocyte-common (L-C) antigen of rat.
European Journal of Immunology 10; 737-744

Davson J, Ball J, Platt R.
The kidney in periarteritis nodosa.
Quart.J.Med. (1948): 17; 175-202

DeRemee RA, McDonald TJ, Weiland LH.
Wegener's granulomatosis: Observations on treatment with antimicrobial agents.
Mayo.Clin.Proc. (1985): 60; 27-32

DeRemee RA, McDonald TJ, Weiland LH.
Wegener's granulomatosis: Experience with over 100 cases. New Approaches for Treatment.
Sarcoidosis (1986): 3; 143-144

Dewald B, Bretz V, Baggiolini M.
Release of gelatinase from a novel secretory compartment of human neutrophils.
J.Clin.Invest. (1982): 70; 518-525

Dighiero G, Guilbert B, Avrameas S.
Naturally occurring antibodies against nine common antigens in human sera. II - high incidence of monoclonal Ig exhibiting antibody activity against actin and tubulin and sharing antibodies specificities with natural antibodies.
J.Immunol. (1982): 128; 2788-2792

Dighiero G, Lymberi P, Guilbert B et al.
Natural Autoantibodies Constitute a Substantial Part of Normal Circulating Immunoglobulins.
Ann.N.Y.Acad.Sci (1986): 475; 135-145

Dighiero CH, Magnac J, De Saint Mantin N, Avrameas S.
Detection of antimitochondrial antibodies by ELISA and Western blot techniques and identification by one and two dimensional gel eletrophoresis of M2 target antigens.
Clin.Exp.Immunol. (1987): 70; 640-648

Dinauer MC, Orkin SH, Brown R, Jesaihs AJ, Parkos CA.
The glycoprotein encoded by the X-linked chromosome granulomatous disease locus is a component of neutrophil cytochrome B complex.
Nature (1987): 327, 717-720

Donald KJ, Edwards RL, McEvoy JSS.
An ultrastructural study of the pathogenesis of tissue injury in limited Wegener's granulomatosis.
Pathology (1976(: 8; 161-169

Douglas AC, Anderson TJ, MacDonald MK, Simpson JG.
Midline and Wegener's granulomatosis.
Ann.N.Y.Acad.Sci (1976): 278; 618-635

Drachman DB, Adams RN, Josifek LF, Self SG.
Functional activities of autoantibodies to acetylcholine receptors and the clinical severity of myasthenia gravis.
N.Engl.J.Med. (1982): 307; 769-775

Dunnill MS.
In: Pathological Basis of Renal Disease (2nd Edition), Balliere Tindall, Oxford, (1984) pp 189-205.

Elkon KB, Sutherland DC, Rees AJ, Hughes GRV, Batchelor JR.
HLA - antigen frequencies in systemic vasculitis: Increase in HLA DR2 in Wegener's granulomatosis.
Arthritis. Rheum. (1983): 26; 102-105

Fauci AS, Wolff SM, Johnson JS.
Effect of cyclophosphamide upon the immune response in Wegener's granulomatosis.
N.Engl.J.Med. (1971): 285; 1493-1496

Fauci AS, Wolff SM.
Wegener's Granulomatosis: Studies in 18 patients and a review of the literature.
Medicine (1973): 52; 535-561

Fauci AS.
Granulomatous Vasculitides: distinct but related.
Ann.Intern.Med. (1977): 87; 782-783

Fauci AS, Wolff SM.
Wegener's granuloma and related diseases. In: Dowling HF ed.
Disease-a-Month, vol 23, Year Book Medical Publishers, Chicago, (1977): 10-36

Fauci AS.
The spectrum of vasculitis - clinical pathologic immunologic and therapeutic considerations.
Ann.Intern.Med. (1978): 89; 660-676

Fauci AS, Haynes BF, Katz P, Wolff SM.
Wegener's Granulomatosis: Prospective clinical and therapeutic experience with 85 patients for 21 years.
Ann.Intern.Med. (1983): 98; 76-85

Feehally J.
Immune mechanisms in glomerular IgA deposition.
Nephrol.Dial.Transpl. (1988): 4; 361-378

Feinberg R.
The protracted superficial phenomenon in Wegener's granulomatosis.
Human Pathol. (1981): 12; 458-467

Ferrario F, Castiglione A, Colasanti G, Barbiano De Belgioioso G, Bertoli S, D'Amico G & Nava S.
The detection of monocytes in human glomerulonephritis.
Kidney Intenational (1985): 28; 513-519

Freedman P, Peters JH, Kark RM.
Localisation of gamma globulin in the diseased kidney.
Arch.Intern.Med. (1960): 105, 524-535

Gocke DJ, HsuK, Morgan C et al.
Association between polyarteritis and Australia antigen.
Lancet (1970):ii; 1149-1153

Godman GC, Churg J.
Wegener's granulomatosis. Pathology and a review of the literature.
Arch.Pathol. (1954): 58; 533-553

Goldschmeding R, Tetteroo PAT, Van der Borne AEG.
Antineutrophil cytoplasm antibodies in Wegener's granulomatosis are not directed against alkaline phosphatase.
Lancet (1987): i; 1489

Goldschmeding R, Tetteroo PAT, Van der Borne AEG.
Wegener's granulomatosis specific autoantibodies (ACPA) characterisation of the antigen.
Kidney Intern. (1987): X; 779A

Goldschmeding R, Tetteroo PAT, The TH Van der Borne AEG.
Wegener's granulomatosis specific autoantibodies (ACPA) characterisation of the antigen.
Kidney Intern. (1987): 32; 779A

Grabar P.
Hypothesis: Autoantibodies and immunologic theories: an analytic review.
Clin.Immunol.Immunopath. (1975): 4; 453-466

Gross WL, Ludemann G, Kiefer G, Lekmann H.
Anticytoplasmic antbodies in Wegener's granulomatosis.
Lancet (1986): i; 806

Gross WL, Ludemann G, Schroder JM.
Antineutrophil cytoplasm antibodies in Wegener's granulomtosis are not directed against alkaline phosphatase.
Lancet (1987): i; 1488-1489

Guilbert B, Dighiero G, Avraneas S.
Naturally occurring antibodies against nine common antigens in human sera. I - Detection, isolation and characterisation.
J.Immunol. (1982): 182; 2779-2787

Hall SL, Miller LC, Duggan E et al.
Wegener's granulomatosis in pediatric patients.
J.Padiatrics (1985): 106; 739-744

Handcock WW & Atkins RC.
Cellular composition of crescents in human rapidly progressive glomerulonephritis identified using monoclonal antibodies.
American Journal of Nephrology (1984): 4; 177-181

Harlan JM, Killen PD, Harker La, Striker GE.
Neutrophil mediated endothelial injury in vitro.
J.Clin.Invest. (1981): 68; 1394-1403

Harrison DJ, Thomson D, MacDonald MK.
Membranous glomerulonephritis.
J.Clin.Path. (1986): 39; 167-171

Hensley MJ, Feldman MT, Lezarus JM & Galvanek EG.
Diffuse pulomonary haemorrhage and rapidly progressive renal failure. An uncommon presentation of Wegener's granulomatosis.
Am.J.Med.(1977):66; 894-898

Henson PM, Cochrane CG.
Acute immune complex disease in rabbits: the role of complement and of a leukocyte dependent release of vasoactive amines from platelets.
J.Exp.Med. (1971): 133; 554-571

Heptinstall RH.
In: Pathology of the Kidney (3rd edition) Little, Brown and Co, Boston/Toronto, (1983) pp 793-838

Heyderman E, Steele K & Ormerod MG.
A new antigen on the epithelial membrane: its immunoperoxidase localisation in normal and neoplastic tissue.
Journal of Clinical Pathology (1979): 32; 35-39

Hird CRK, Winearls CG, Lockwood CM et al.
Objective monitoring of activity in Wegener's granulomatosis by measurement of serum C-reactive protein concentration.
Clin.Nephrol. (1984): 21; 341-345

Holdsworth SR, Allen DE, Thomson NM, Glasgow EF & Atkins RC.
Histochemistry of glomerular cells in animal models of crescentic glomerulonephritis.
Pathology (1980) 12; 339-346

Holmberg D, Coutinho A.
Natural antibodies and autoimmunity.
Immunol.Today (1985): 6; 356-357

Holthoffer H, Miettinen A, Pasivuo R, Lehto VP, Linder E, Alfthan O & Ultranen I.
A fluorescence microscopic study with kidney-specific antibodies, anti-intermediate filament antibodies, and lectins.
Laboratory Investigation (1983): 49; 317-326

Horn RG, Fauci AS, Rosenthal AS. Wolff SM.
Renal biopsy pathology in Wegener's granulomatosis.
Am.J.Path. (1974): 74; 423-433

Howell SB, Epstein WV.
Circulating immunoglobulin complexes in Wegener's granulomatosis.
Am.J.Med. (1976): 60; 259-268

Howie AJ.
Epithelial membrane antigen in normal and proteinuric glomeruli and in damaged proximal tubules.
Journal of Pathology (1986): 148; 55-60

Hu CH, O'Lauglin S, Winkelmann RK.
Cutaneous manifestations of Wegener's granulomatosis.
Arch.Dermatol. (1977): 113; 175-182

Israel HL, Patchefsky AS, Saldana MJ.
Wegener's granulomatosis, lymphatoid granulomatosis and benign lymphocytic angitis and granulomatosis of the lung: recognition and treatment.
Ann.Intern.Med. (1977): 87; 691-699

Juncos LI, Alexander RW, Marburg TC.
Intravascular Clotting Preceeding Crescent Formation in a Patient with Wegener's Granulomatosis and Rapidly Progressive Glomerulonephritis.
Nephron (1979): 24; 17-20

Katz P, Alling DW, Haynes BF, Fauci AS.
Association of Wegener's granulomatosis with HLA-B8.
Clin.Immunol.Immunopathol. (1979): 14; 268-270

Kjellstrand CM, Simmons RL, Uranja M et al.
Acute fulminant Wegener's granulomatosis.
Arch.Intern.Med. (1974): 134; 40-43

Klinger H.
Grenzformen der periarterites nodsa Frankfurter
Zeitschrift Fur Pathologie (1931): 42; 445-480

Kohn LD, Alvarez F, Marocci C et al.
Monoclonal antibody studies defining the origin and properties of autoantibodies in Grave's disease.
N.Y.Acad.Sci. (1986): 475; 157-173

Kyriatsoulis A, Manns M, GerkenG etal.
Distinction between natural and pathological autoantibodies by immunoblotting and densitometric subtraction: Liver kidney microsomal antibody (LKM) positive sera identify multiple antigens in human liver disease.
Clin.Exp.Immunol. (1987): 70; 53-60

Laemmli UK.
Cleavage of structural proteins during assembly of the head of bacteriophage T4.
Nature (1970): 227: 680-685

Leavitt RY, Fauci AS.
State of the Art - Pulmonary Vasculitis.
Am.Rev.Respir.Dis. (1986): 134; 149-166

Lockwood CM, Bakes D, Jones S et al.
Association of Alkaline Phosphatase with an autoantigen recognised by circulating anti-neutrophil antibodies in systemic vasculitis.
Lancet (1987): i; 716-720

Loke SL, Neckers LM, Schwab G, Jaffe ES.
C-myc protein in normal tissue. Effects of fixation on its apparent subcellular distribution.
Am.J.Pathol. (1988): 131; 29-37

Ludemann G, Gross WL.
Autoantibodies against cytoplasmic structures of neutrophil granulocytes in Wegener's granulomatosis.
Clin.Exp.Immunol.(1987): 69; 350-357

Lutz HU, Wipf G.
Naturally occurring autoantibodies to skeletal proteins from human red blood cells .
J.Immunol. (1982):128; 1695-1699

Madaio MP, Schattner A, Shattner M, Schwartz RS.
Lupus serum and normal human serum contain anti-DNAA antibodies with the same idiotypic marker.
J.Immunol. (1986): 137; 2535-2540

Magil AB.
Histogenesis of glomerular crescentts.
American Journal of Pathology (1985): 120; 222-229

Magil A, Calderon L, Gershon S.
Demonstration of immune complex deposits by electron microscopy and immunofluorrescence in a fatal case of hypersensitivity angiitis.
Can.Med.Assoc.J. (1978): 119; 751-752

Magil AB & Wadsworth LD.
Monocyte involvement in glomerular crescents. A histochemical and ultrastructural study.
Laboratory Investigation (1982): 47; 160-166

Malech HL, Gallen JI.
Neutrophils in human diseases.
New.Eng.J.Med. (1987): 317, 687-693

Marshall RJ & MacIver AG.
The monocyte/macrophage population of the normal human kidney.
Journal of Pathology (1984): 143; 274-280

Mason DY & Taylor CR.
The distribution of muramidase (Lysozyme) in normal tissues.
Journal of Clinical Pathology (1975): 28; 124-134

Matthay RA, Bromberg SI, Putman CE.
Pulmonary Renal Syndrome - A review.
Yale.J.Biol.Med. (1980): 53; 497-523

McCluskey RT, Feinberg O.
Vasculitis in primary vasculitis, granulomatoses and connective tissue diseases.
Hum.Pathol. (1983): 14; 305-315

McIntosh RM, Tinglof F, Kaufman D, et al.
Immunohistology in renal disease.
Q.J.Med. (1971): 159; 385-390

Min K, Gyorkey P, Yium J & Eknoyan G.
The morphogenesis of glomerular crescents in rapidly progressive glomerulonephritis.
Kidney Interrnational (1974): 5; 47-56

Morita T, Suzuki Y & Churg J.
Structure and development of the glomerular crescent.
American Journal of Pathology. (1973): 72; 349-368

Nolasco F, Cameron JS, Reuben R, Hartley B & Welsh K.
A shared antigenic determinant present in natural killer cells, renal tubular cells and fibrocellular crescents.
Proceedings of the IXth International Congress of Nephrology, p 485. Los Angeles, California (1984)

Noritake DT, Weiner SR, Basset LW, Paulus HE, Weisbart R.
Rheumatic manifestations of Wegener's granulomatosis.
J.Rheumatol. (1987): 145; 949-951

Norton WL, Suki W, Strunk S.
Combined corticosteroid and azathioprine therapy in two patients with Wegener's granulomatosis.
Arch.Intern.Med. (1968): 121; 554-560

Novak RF, Christiansen RG, Sorensen ET.
The acute vasculitis of Wegener's granulomatosis in renal biopsies.
Am.J.Clin.Pathol. (1982): 78; 367-371

Ohyashiki K, Kocova M, Ryu DH et al.
Secondary acute myeloblastic leukaemia with a Ph translocation in a treated Wegener's granulomatosis.
Cancer Genet.Cytogenet. (1986): 19; 331-333

Parleviet KJ, Hengen-Logmans SC, OE PL, Bronsveld W, Balm AJM, Donker AJM.
Antibodies to components of neutrophil cytoplasm: A new diagnostic tool in patients with Wegener's granulomatosis and systemic vasculitis.
Q.J.Med. (1988): 249; 55-63

Paronetto F, Strauss L.
Immunocytochemical observations in periarteritis nodosa.
Ann.Intern.Med. (1962): 56: 289-296

Patek CE, Vornhagen R, Rink N, Clayton RM.
Developmental changes in membrane protein expression by chick lens cells in vivo and in vitro and the detection of Main Intrinsic Polypeptide (MIP).
Exp.Eye.Res. (1986): 43; 29-40

Pavane-Macaluso M.
Tissue mast cells in renal diseases.
Acta.Pathol.Microbiol.Scaand. (1960): 50; 337-346

Pinching AJ, Lockwood CM, Pussell BA et al.
Wegener's granulomatosis: Observations on 18 patients with severe renal disease.
Q.J.Med. (1983): 208; 425-460

Rasmussen N, Borregaard N, Wiik A.
Antineutrophil cytoplasm antibodies in Wegener's granulomatosis are not directed against alkaline phosphatase.
Lancet (1987): i; 1488

Rasmussen N, Wiik A, Hoier-Madsen M, Borregaard N, Van der Woude FJ.
Antineutrophil cytoplasm antibodies.
Lancet (1988): i; 706-707

Reza MJ, Dornfeld L, Goldbergh LS, Bluestone R, Pearson CM.
Wegener's granulomatosis: Long-term follow up of patients treated with cyclophosphamide.
Arthritis Rheum. (1976): 18; 501-506

Rich AR.
The role of hypersensitivity in polyarterits nodosa as indicated by serum cases developing during serum sickness and sulfonamide therapy.
Bull.John Hopkins Hosp. (1942a): 71; 123-140

Rich AR.
Additional evidence of the role of hypersensitivity in the aetiology of polyarteritis nodosa: another case associated with a sulfonamide reaction.
Bull.John Hopkins Hosp. (1942b): 71; 375-379

Roback SA, Herdman RC, Hoyer J et al.
Wegener's granulomatosis in a child. Observations on pathogenesis and treatment.
AM.J.Diseases Child. (1969): 118; 608-614

Robbins SL, Cotran RS, Kumar V.
In:Pathological basis of disease (3rd edition).
WB Saunders Co (1984): pp519-528

Rodbard D.
Statistical quality control and routine data processing for radioimmunoassays and immunoradiometric assays.
Clin.Chem. (1974): 20; 1255-1270

Roitt IM, Cooke A.
The role of autoantigen in autoimmunity.
Immunol.Letters (1987): 16; 259-264

Ronco P, Verroust P, Mignon F et al.
Immunopathological studies of polyarteritis nodosa and Wegener's granulomatosis: a report of 43 patients with 51 renal biopsies.
Q.J.Med. (1983): 206; 212-223

Rose NR, Herskowitz A, Newman DA, Neu N.
Autoimmune myocarditis: a paradigm of post-infection autoimmune disease.
Immunol.Today (1988): 9; 117-120

Salter DM, Krajewski AS & Dewar AE.
Immunohistochemical staining of Hodgkin's lymphoma with monoclonal antibodies specific for the leucocyte-common antigen.
Journal of Pathology (1985): 146; 345-353

Sart GR, Ucci AA, Meares EM.
Renal immunoblastic sarcoma complicating immunosuppressive therapy for Wegener's granulomatosis.
Urology, (1983): 21; 632-634

Savage COS, Winearls CG, Evans DJ, Rees AJ, Lockwood CM.
Microscopic polyarteritis: Presentation, pathology and prognosis.
Q.J.Med. (1985): 220; 467-483

Savage COS, Winearls CG, Jones S, Marshall PD, Lockwood CM.
Prospective study of radioimmunoassay for antibodies against neutrophil cytoplasm in diagnosis of systemic vasculitis.
Lancet, (1987): ii; 1389-1393

Sayers TJ, Binder T, Berg PA.
Heterogeneity of the mitochondrial antibodies: characterisation and separation of the antigen associated with pseudolupus syndrome.
Clin.Exp.Immunol. (1979): 37; 68-75

Schiffer MS, & Michael AF.
Renal cell turnover studies by Y chromosome (Y body) staining of transplanted human kidney .
Journal of Laboratory and Clinical Medicine (1978): 92; 841-847

Schultheiss HP, & Bolte HD.

J.Mol.Cell.Cardiol. (1985): 17; 603-617

Schultheiss HP & Schwimnbeck P.
Autoantibodies to the adenine nucleotide translocator (ANT) in myocarditis (MC) - prevalence, clinical correlates and diagnostic value.
Circulation, (1986): 74; 142A

Scott J, Finch LD.
Wegener's granulomatosis presenting as gingivitis. Review of the clinical and pathological features and report of a case.
Oral Surg. (1982): 34; 920-933

Segal AW, & Peters TJ.
Analytic subcellular fractionation of human granulocytes with special reference to localisation of enzymes involved in microbicidal mechanisms.
Clin.Sc.Mol. (1977): 52 (4); 429-442

Segal AW, Heyworth PG, Cockcroft S and Barrowman MM.
Stimulated neutrophils from patients with autosomal recessive chronic granulomatous disease fail to phosphorylated Mr 44,000 protein.
Nature, (1985): 316; 547-549

Segrest JP, & Jackson RL.
Molecular weight determination of glycoproteins by polyacrylamide gel electrophoresis in sodium dodecyl sulphate.
Methods Enzymol (1972): 28; 54-63

Seligmann M & Brouet JC.
Antibody activity of human myeloma globulins.
Semin. Hematol. (1973): 10; 163-177

Serra A, Cameron JS, Turner DR et al.
Vasculitis affecting the kidney: Presentation, histopathology and long-term outcome.
Quart.J.Med. (1984): 210; 181-207

Sharp GC, Irvin WS, Tan EM, et al.
Mixed connective tissue disease - An apparently distinct rheumatic disease syndrome associated with a specific antibody to an extractable nuclear antigen (ENA).
Am.J.Med. (1972): 52; 148-159

Singh S, Yap PL, Douglas AC.
Immunological parameters in midline granuloma.
J.Clin.Lab.Immunol. (1982): 9; 71-74

Sinico RA, Fornasieri A, Oreni N, et al.
IgA rheumatoid factor in idiopathic IgA mesangial nephropathy (Bergers disease).
Nephrol.Dial.Transpl. (1986): 1; 77A

Smedley LA, Torresen MG,Sandhauss RA et al.
Neutrophil mediated injury to endothelial cells. Enhancement by endotoxin and essential role of neutrophil elastase.
J.Clin.Invest. (1986): 77; 1233-1243

Stanziale P, Balleta M, Exposito A et al.
Predictive factors of renal failure progression in membranous glomerulonephritis (MGN).
Kidney Intern. (1985): 28; 259A

Stemshorn BW, Buckley DJ, SC Amour G et al.
A computer-interfaced photometer and systemic spacing of duplicates to control within - plate enzyme - immunoassay variation.
J.Immunol.Methods. (1983): 61; 367-375

Sternberger LA, Hardy PH Jr, Cuculis JJ & Meyer HG.
The unlabelled antibody enzyme method of immunohistochemistry. Preparation and properties of soluble antigen-antibody complex (horse radish peroxidse anti-horse radish peroxidase) and its use in identification of spirochaetes.

Sun TT, Shih C, Green H.
Keratin cytoskeletons in epithelial cells of internal organs.
Proc.Nat.Acad.Sci. (USA) (1979): 76; 2812-2817

Tan EM.
Relationship of nuclear staining patterns with precipitating antibodies in systemic lupus erythematosus.
J.Lab.Clin.Med. (1967): 70; 800-812

Tijssen P.
Localisation and identification of electrophoretically separated antigens after their transfer from gels to membrane. In: Burden RH, Van Knippenberg PH. Laboratory techniques and molecular biology Vol 15 - practice and theory of enzyme immunoassays.
The Netherlands, Elsewier Science Publishers (1987): 423-447

Van der Woude FJ, Hoorntje SJ, Weening JJ, Van Overbeck JJM, Van der Herm GK.
Renal involvement in Wegener's granulomatosis. Report of three universal cases.
Nephron (1982): 32; 185-187

Van der Woude FJ, Rasmussen N, Lobatto S, etal.
Autoantibodies against neutrophils and monocytes: tool for diagnosis and marker of disease activity in Wegener's granulomatosis.
Lancet, (1985): i; 425-429

Venning MC, Arfeen S, Bird AG.
Antibodies to neutrophil cytoplasmic antigen in systemic vasculitis.
Lancet (1987): ii; 850

Vincent A.
Immunology of acetylcholine receptors in relation to myasthenia gravis.
Physiol.Rev. (1980): 60; 756-824

Walport M.
The identification of autoantigens, autoantibodies and their idiotypes.
Br.J.Rheum. (1987): 26; 161-167

Walton EW.
Giant cell granuloma of the respiratory tract (Wegener's granulomatosis).
Br.Med.J. (1958): 2; 265-270

Watanabe T, Najefuchi Y, Yoshikawa Y et al.
Renal papillary necrosis associated with Wegener's granulomatosis.
Human Pathol. (1983): 14; 551-557

Wathen CG, Bell D, Harrison DJ, Jackson M, Dawes J.
Serum human neutrophil elastase is increased in patients with active Wegener's granuloma.
Thorax (1987): 42; 750

Wegener F.

Uber generalisierte, septiscle Geffasser Krankungen Verh Dtsch Ges Path (1963): 29; 202-209

Wegener F.

Uber eine eigenartige rhinogene Granulomatose mit besender Berligurg des Arteriencystems und der Nieren.

Beitr Path (1939): 102; 36-38

Weiss MA, Grissman JD.

Renal biopsy findings in Wegener's granulomatosis: segmental necrotising glomerulonephritis with glomerular thrombosis.

Hum.Pathol. (1984): 15; 943-956

Weiss SJ, Regiani S.

Neutrophil degrade subendothelial matrices in the presence of alpha-1-proteinase inhibitor. Co-operative use of lysosomal proteinases and oxygen metabolites.

J.Clin.Invest. (1984): 73; 1297-1303

Wheeler GE.

Cyclophosphamide associated leukemia in Wegener's granulomatosis.

Ann.Intern.Med. (1981): 94; 361-362

Wheeler J, Simpson J, Morley AR.
Routine and rapid enzyme linked immunosorbent assays for circulating anti-glomerular basement membrane antibodies.
J.Clin.Path. (1988): 41; 163-170

Whitaker AN, Emerson BT, Bunce IH et al.
Reversal of renal failure in Wegener's granulomatosis by heparin.
Amer.J.Med.Sci. (1973): 265; 399-406

Wick G, Kromer G, Neu N, et al.
The multifactorial pathogenesis of autoimmune disease.
Immunol.Letters (1987): 16; 249-258

Williams ARW, Wyllie AH, Piris J.
Immunohistochemical demonstration of p62 myc oncogene product in human colonic neoplasms.
J.Pathol. (1987): 152; 234A

Wolff SM, Fauci AS, Horn RG, Dale DC.
Wegener's granulomatosis.
Ann.Intern.Med. (1974): 81; 513-525

APPENDIX

PUBLICATIONS FROM THE THESIS

Some of this thesis has been published as refereed papers.

Papers:

1. Harrison DJ, MacDonald MK.
   The Origin of Cells in the Glomerular Crescent Investigated by the Use of Monoclonal Antibodies.
   Histopathology (1986)

2. Harrison DJ, Neary C, Wathen CG.
   Distribution of IgE-Bearing Cells in Renal Biopsies in Wegener's Granulomatosis.
   Nephrol. Dial. Transplant. (1988)

3. Harrison DJ, Simpson R, Neary C, Wathen CG.
   Renal Biopsy and Antineutrophil Antibodies in the Diagnosis and Assessment of Wegener's Granuloma.
   Br. J. Dis. Chest (1988)

4. Harrison DJ, Kharbanda R.
   Autoantibodies to Neutrophil Cytoplasmic Antigens in Systemic Vasculitis have the Same Target Specificity.
   J. Pathol. (1989)

5. Harrison DJ, Simpson R, Kharbanda R, Abernethy V, Nimmo G.
   Antibodies to Neutrophil Cytoplasm Antigens in Wegener's Granulomatosis and Other Conditions.
   Thorax (1989)

Histopathology 1986, **10**, 945–952

The origin of cells in the glomerular crescent investigated by the use of monoclonal antibodies

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HARRISON D.J. & MACDONALD M.K. (1986) *Histopathology* **10**, 945–952

The origin of cells in the glomerular crescent investigated by the use of monoclonal antibodies

A study was made of the cells forming the crescents in human crescentic glomerulonephritis. The investigation was performed using a panel of antibodies with immunoperoxidase techniques in formalin fixed, paraffin embedded renal biopsy material. Some of the cells of glomerular crescents were found to contain cytokeratin intermediate filaments, as did some of the cells of the normal parietal epithelium of Bowman's capsule. Leucocytes were also found in crescents, often in the outer part, and their presence was associated with a mantle of inflammatory cells around the glomerulus. The use of paraffin embedded rather than frozen tissue allowed better histological assessment than has been possible in previous studies. The glomerular crescents appeared to be primarily epithelial in origin, with leucocytes contributing to the overall inflammatory response.

Keywords: crescentic glomerulonephritis, monoclonal antibodies, immunoperoxidase

Introduction

Many attempts have been made to determine the histogenesis of the cells found in glomerular crescents, both in animal models and in human biopsy material. Histological and ultrastructural observations have suggested an origin from the parietal epithelial cells of Bowman's capsule (Morita, Suzuki & Churg 1973, Min *et al.* 1974), though not excluding a role for monocytes (Magill & Wadsworth 1982). Glomerular culture and histochemical methods have favoured a monocytic origin (Atkins *et al.* 1976, Holdsworth *et al.* 1980, Ferrario *et al.* 1985), while cytogenetic studies have shown that crescent formation cannot be explained entirely by macrophage migration (Schiffer & Michael 1978). An experimental rabbit model of serum nephritis has demonstrated monocytic involvement (Cattell & Arlidge 1981), but indicated that

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parietal cells are also present, particularly in the late stage of development of crescents (Cattell & Jamieson 1978). More recently, monoclonal antibodies have been used as investigative tools and have suggested that, in cultured glomeruli, epithelial cells make little contribution (Handcock & Atkins 1984). In frozen sections, use of a monoclonal antibody against cytokeratin intermediate filaments, a constituent of epithelial cytoplasm (Sun, Shih & Green 1979, Holthofer *et al.* 1983), provided information that epithelial cells contribute significantly to crescent formation (Magill 1985), although macrophages were also detected by non-specific esterase histochemistry. Fibrocellular crescents have been shown to possess an antigenic determinant in common with renal tubular cells and natural killer cells, whereas the cells of cellular crescents appeared to bear mainly macrophage markers (Nolasco *et al.* 1984). This is supported by the work of Cattell & Jamieson (1978) on an experimental rabbit model.

This present study investigates the histogenesis of crescents in paraffin embedded human renal biopsies, by the use of monoclonal antibodies against cytokeratin, leucocyte common antigen and epithelial membrane antigen, and of a polyclonal antibody against muramidase, a marker of monocytes/macrophages (Mason & Taylor 1975); this last antibody has previously been used to investigate the significance of monocytes in normal kidney (Marshall & MacIver 1984).

Materials and methods

SELECTION OF CASES

Eighteen percutaneous or open renal biopsies of patients with crescentic glomerulonephritis were available for study. Sections of formalin fixed, paraffin embedded tissue were examined by light microscopy, and frozen sections were used for immunofluorescence studies with conventional methods. For a diagnosis of crescentic glomerulonephritis it was necessary for at least 70% of glomeruli to possess crescents. Control renal tissue was obtained from five nephrectomy specimens, removed because of renal carcinoma: none of these showed any significant glomerular or tubulo-interstitial lesion. The diagnoses made at time of biopsy, in addition to crescentic glomerulonephritis, were (i) anti-glomerular basement membrane disease, on the basis of linear immunofluorescence staining; (ii) probable microscopic polyarteritis, with segmental glomerular lesions; or (iii) diffuse endocapillary proliferative glomerulonephritis; in six cases no underlying disease was established.

REAGENTS AND TECHNIQUES

The antibodies used were a polyclonal anti-muramidase raised in rabbit (Dako Antimuramidase, Dako Ltd, High Wycombe, UK) (Mason & Taylor 1975) and mouse monoclonal antibodies against epithelial membrane antigen (EMA), which is a surface marker on many epithelial cells (Dako EMA, Dako Ltd) (Heyderman, Steele & Ormerod 1979), against cytokeratin (CK), which is a cytoplasmic intermediate filament (Dako PKK1, Dako Ltd) (Holthofer *et al.* 1983), and against leucocyte

Table 1. Tabulation of crescentic glomerulonephritis (CGN) cases giving diagnosis associated with CGN, the number of crescents examined, the number of glomeruli containing crescents with varying grades of cytokeratin positivity and the average number (and range) of leucocytes in tuft capillaries and crescents

Case no.	Diagnosis associated with CGN	No. of crescents examined	No. of glomeruli with cytokeratin in crescents according to grade of positivity				Average no. of leucocytes per tuft (range)	Average no. of leucocytes per crescent (range)
			0	1	2	3		
1	PA	13	8	5	0	0	2.4 (1–5)	2 (0–5)
2	NL	13	6	4	3	0	0.7 (0–5)	0.5 (0–3)
3	NL	4	3	1	0	0	2.5 (0–5)	No tissue
4	NL	10	9	0	1	0	0.4 (0–2)	0
5	aGBM	8	8	0	0	0	0.4 (0–2)	0.3 (0–2)
6	PA	13	8	0	2	3	5.5 (2–11)	2.0 (0–6)
7	NL	9	2	3	3	1	2.0 (0–5)	3.0 (0–9)
8	DEPGN	9	9	0	0	0	0.3 (0–2)	0
9	DEPGN	9	7	2	0	0	2.8 (1–3)	0.3 (0–1)
10	aGBM	2	Insufficient tissue				5.0 (1–2)	6.0 (6)
11	aGBM	3	2	0	1	0	2.5 (1–5)	No tissue
12	aGBM	7	7	0	0	0	1.4 (0–4)	0.1 (0–1)
13	NL	6	5	1	0	0	1.0 (0–2)	0
14	NL	3	3	0	0	0	0.7 (0–1)	0
15	aGBM	5	Insufficient tissue				1.8 (0–4)	0.9 (0–2)
16	PA	3	2	0	1	0	1.6 (0–3)	No tissue
17	aGBM	9	8	0	1	0	1.9 (0–5)	0.9 (0–3)
18	PA	24	7	11	6	0	1.2 (0–3)	1.5 (0–4)

PA = Probable microscopic polyarteritis; NL = no obvious lesion; aGBM = anti-glomerular basement membrane disease; DEPGN = diffuse endocapillary proliferative glomerulonephritis.

common antigen (LCA), which is present on the surface of most lymphoid and many monocytic cells (Dako LC, Dako Ltd) (Dalchau, Kirkley & Fabre 1980). Immunoperoxidase methods were employed, and were the standard techniques for paraffin sections (Sternberger *et al.* 1970, Salter, Krajewski & Dewar 1985), sections being trypsinized before incubation with the primary antisera.

ASSESSMENT

The number of crescents per case was recorded and ranged from 2 to 24 (Table 1). The cells staining with the antibodies were noted and a count of the number of LCA positive cells in the glomerular tuft and the crescents was made. Cytokeratin positivity in the crescent was graded on a four point scale (0–3) where 0 indicated no positive staining, and 3 signified that more than 50% of the crescent cells were positive. No attempt was made to quantify EMA and muramidase positivity, but their distribution was noted. Assessment was made by two observers and was found to be readily reproducible.

Results

CONTROL KIDNEY

Muramidase

This was distributed in the cells of most convoluted tubules, in monocytes and in neutrophils. Glomerular capillaries contained an average of 5.5 intraluminal positive cells per tuft (range 0–18).

Epithelial membrane antigen

Cells of collecting tubules and many convoluted tubules reacted positively. Occasional glomeruli contained very small foci of positive staining, as previously described by Howie (1986).

Cytokeratin

Cells of collecting tubules were strongly positive and many convoluted tubules also reacted, although less intensely. No elements of the glomerular tuft reacted, but some parietal epithelial cells of Bowman's capsule did react strongly (mean 32%, range 0–100%) (Figure 1).

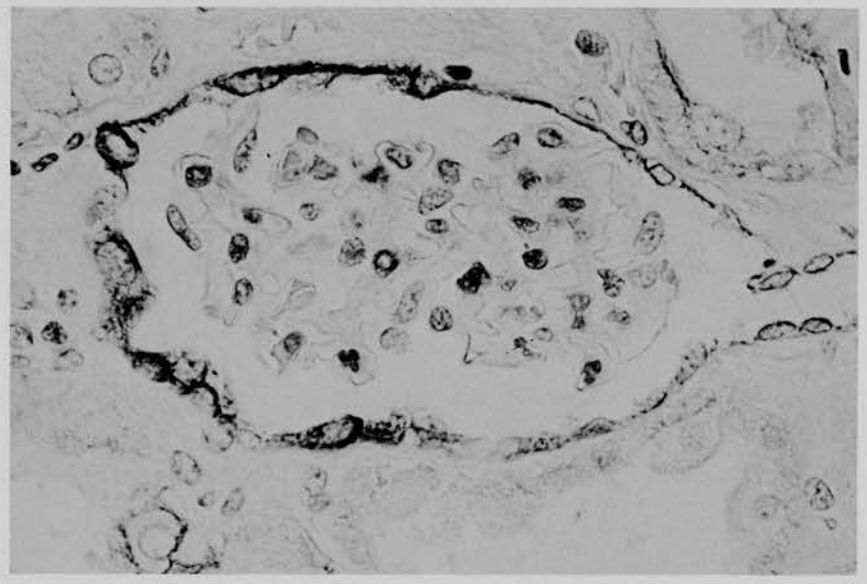

Figure 1. A normal glomerulus showing a positive reaction for cytokeratin by most parietal epithelial cells. Note the variation in reaction intensity. PAP. ×500.

Leucocyte common antigen

Monocytes, interstitial and intravascular lymphocytes and monocytes reacted and each glomerular tuft contained an average of three positive intraluminal cells (range 0–9). No renal elements and, in particular, no cells in mesangial regions reacted.

CRESCENTIC GLOMERULONEPHRITIS

Significant results are summarized in Table 1. Crescents were also classed as being cellular, fibrocellular or fibrous.

Muramidase

The anti-muramidase antibody reacted with, in addition to convoluted tubules, some capsular parietal epithelial cells in glomeruli in which crescents were not present, many intra- and extravascular polymorphs and macrophages, and many crescent cells. The reaction of crescent cells was not uniform; a few cells reacted strongly, more reacted weakly, but most were negative. Quantification was not attempted.

Epithelial membrane antigen

The antibody reacted with some convoluted tubules and collecting tubules but no crescents, with the exception of three cells in a glomerular crescent in one case.

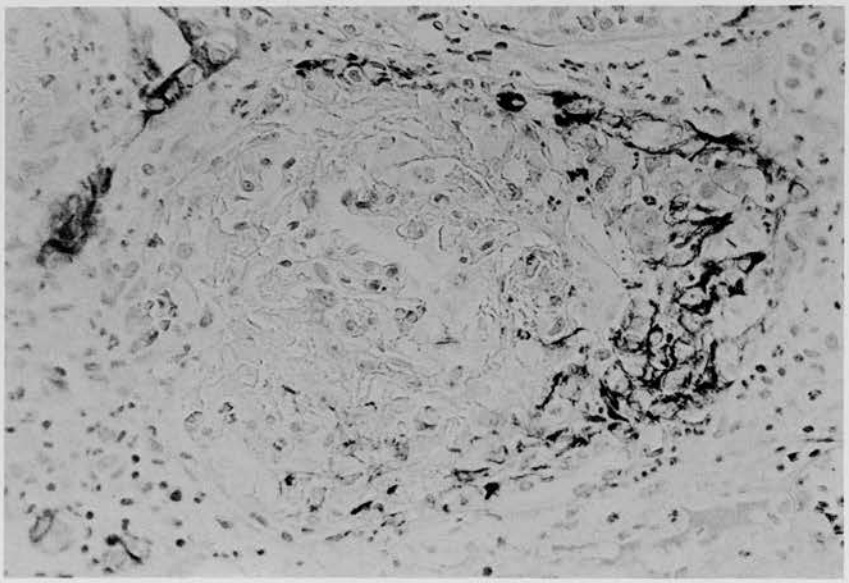

Figure 2. A glomerulus containing a cellular crescent which is strongly positive for cytokeratin. The glomerular tuft does not react but some nearby tubular cells are positive. PAP. ×320.

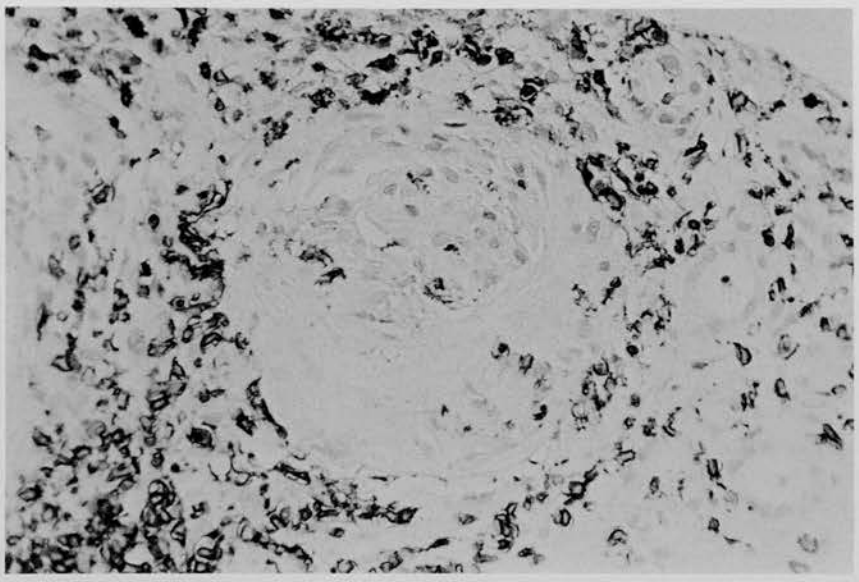

Figure 3. A fibrocellular crescent, compressing the glomerular tuft, surrounded by numerous leucocyte common antigen positive mononuclear cells. Some reacting cells are present in the crescent and also in the lumina of tuft capillaries. ×320.

Cytokeratin

Overall, 34% of crescents contained CK positive cells. The median grade of positive crescents with reacting cells was grade 1 (mean 1.5). Fibrous crescents did not react with the antibody, and in epithelial and fibroepithelial crescents the intensity of reaction varied considerably within a single crescent (Figure 2). Crescents with marked mitotic activity tended to have a higher proportion of positive cells, although it was not possible to determine the nature of the mitotic cells. It was noted that on occasions CK positive cells were concentrated on the aspects of the crescent adjacent to the glomerular tuft.

Leucocyte common antigen

Glomerular capillaries contained an average of 1.7 positive cells per glomerulus and these were all intraluminal (range 0–11). Thirty per cent of crescents contained positive cells (mean 1.1 cells per crescent) and these tended to be in the outer part of the crescent. In eight cases, positive cells in the crescent were associated with a ring of positive cells lying just outside Bowman's capsule suggesting that the intracrescentic cells may have entered through Bowman's capsule (Figure 3).

Discussion

We have shown that a small proportion of crescent cells contain cytoplasmic cytokeratin, and are therefore likely to be epithelial in origin (Sun *et al.* 1979, Holthofer *et*

al. 1983). Thirty-two per cent of normal parietal cells have been shown to contain cytokeratin and so we confirm that the epithelial cells of the glomerular crescent are derived from the parietal epithelium of Bowman's capsule. The presence of large numbers of cytokeratin negative cells in the crescent is in keeping with the intermittent demonstration of cytokeratin in the parietal epithelium of the normal Bowman's capsule. This supports the work of Magill (1985) using frozen material, and of those who have used electron microscopy to demonstrate an epithelial origin (Morita *et al.* 1973, Min *et al.* 1974, Magill & Wadsworth 1982). Our findings are not in disagreement with previous studies on cultured glomeruli (Atkins *et al.* 1976, Holdsworth *et al.* 1980, Handcock & Atkins 1984) which demonstrated the importance of monocytes, because these studies did not specifically investigate parietal epithelial cell involvement, but were more concerned with the identification of podocytes (Magill 1985). The muramidase positive staining seen in some parietal epithelial cells in crescentic glomerulonephritis is difficult to explain. One may postulate that these cells either produce muramidase normally in small quantities, with the enhancement of production in crescentic glomerulonephritis, or that muramidase is excreted into Bowman's capsule and reabsorbed leading to a non-specific reaction (Balazs & Roepke 1966).

Leucocytes can be identified in crescents, as others have shown, by histochemical or immunohistochemical methods (Atkins *et al.* 1976, Holdsworth *et al.* 1980, Handcock & Atkins 1984, Nolasco *et al.* 1984, Magill 1985, Ferrario *et al.* 1985). These were found frequently in the outer part of the crescent associated with leucocytes outside Bowman's capsule, in which there are known to be breaks in crescentic glomerulonephritis (Bohman, Olsen & Peterson 1974). Nolasco and his co-workers (1984) showed that, whereas cells of cellular crescents bear macrophage markers, the cells of fibrocellular crescents react with the monoclonal antibody Leu 7 in a manner similar to renal tubular cells. This suggests an epithelial origin for the cells of fibrocellular crescents.

The controversy surrounding the histogenesis of crescents has not been completely resolved, but we have confirmed that both mononuclear leucocytes and epithelial cells are present. This is consistent with the simple proposition that crescents are fundamentally epithelial in origin, and that leucocytes are entering the crescents as part of the overall reactive inflammatory process.

References

Atkins R., Glasgow E.F., Holdsworth S.R. & Matthews F.E. (1976) The macrophage in human rapidly progressive glomerulonephritis. *Lancet* **i,** 830–832

Balazs T. & Roepke R.R. (1966) Lysozymuria induced in rats by nephrotoxic agents. *Proceedings of the Society of Experimental Biology and Medicine (New York)* **123,** 380–385

Bohman S-O., Olsen S. & Peterson V.P. (1974) Glomerular ultrastructure in extracapillary glomerulonephritis. *Acta Pathologica Microbiologica Scandanavia Section A* **82** *(suppl. 249),* 29–54

Cattell V. & Arlidge S. (1981) The origin of proliferating cells in the glomerulus and Bowman's capsule in nephrotoxic serum nephritis: Effects of unilateral irradiation. *British Journal of Experimental Pathology* **62,** 669–675

CATTELL V. & JAMIESON S.W. (1978) The origin of glomerular crescents in experimental nephrotoxic serum nephritis in the rabbit. *Laboratory Investigation* **39,** 584–590

DALCHAU R., KIRKLEY J. & FABRE J.W. (1980) Monoclonal antibodies to a human leucocyte-specific membrane glycoprotein probably homologous to the leucocyte-common (L-C) antigen of rat. *European Journal of Immunology* **10,** 737–744

FERRARIO F., CASTIGLIONE A., COLASANTI G., BARBIANO DE BELGIOIOSO G., BERTOLI S., D'AMICO G. & NAVA S. (1985) The detection of monocytes in human glomerulonephritis. *Kidney International* **28,** 513–519

HANDCOCK W.W. & ATKINS R.C. (1984) Cellular composition of crescents in human rapidly progressive glomerulonephritis identified using monoclonal antibodies. *American Journal of Nephrology* **4,** 177–181

HEYDERMAN E., STEELE K. & ORMEROD M.G. (1979) A new antigen on the epithelial membrane: its immunoperoxidase localisation in normal and neoplastic tissue. *Journal of Clinical Pathology* **32,** 35–39

HOLDSWORTH S.R., ALLEN D.E., THOMSON N.M., GLASGOW E.F. & ATKINS R.C. (1980) Histochemistry of glomerular cells in animal models of crescentic glomerulonephritis. *Pathology* **12,** 339–346

HOLTHOFER H., MIETTINEN A., PAASIVUO R., LEHTO V.P., LINDER E., ALFTHAN O. & ULTRANEN I. (1983) A fluorescence microscopic study with kidney-specific antibodies, anti-intermediate filament antibodies, and lectins. *Laboratory Investigation* **49,** 317–326

HOWIE A.J. (1986) Epithelial membrane antigen in normal and proteinuric glomeruli and in damaged proximal tubules. *Journal of Pathology* **148,** 55–60

MAGILL A.B. (1985) Histogenesis of glomerular crescents. *American Journal of Pathology* **120,** 222–229

MAGILL A.B. & WADSWORTH L.D. (1982) Monocyte involvement in glomerular crescents. A histochemical and ultrastructural study. *Laboratory Investigation* **47,** 160–166

MARSHALL R.J. & MACIVER A.G. (1984) The monocyte/macrophage population of the normal human kidney. *Journal of Pathology* **143,** 274–280

MASON D.Y. & TAYLOR C.R. (1975) The distribution of muramidase (lysozyme) in normal tissues. *Journal of Clinical Pathology* **28,** 124–134

MIN K., GYORKEY F., GYORKEY P., YIUM J. & EKNOYAN G. (1974) The morphogenesis of glomerular crescents in rapidly progressive glomerulonephritis. *Kidney International* **5,** 47–56

MORITA T., SUZUKI Y. & CHURG J. (1973) Structure and development of the glomerular crescent. *American Journal of Pathology* **72,** 349–368

NOLASCO F., CAMERON J.S., REUBEN R., HARTLEY B. & WELSH K. (1984) A shared antigenic determinant present in natural killer cells, renal tubular cells and fibrocellular crescents. *Proceedings of the IXth International Congress of Nephrology,* p. 485. Los Angeles, California

SALTER D.M., KRAJEWSKI A.S. & DEWAR A.E. (1985) Immunohistochemical staining of Hodgkin's lymphoma with monoclonal antibodies specific for the leucocyte-common antigen. *Journal of Pathology* **146,** 345–353

SCHIFFER M.S. & MICHAEL A.F. (1978) Renal cell turnover studies by Y chromosome (Y body) staining of transplanted human kidney. *Journal of Laboratory and Clinical Medicine* **92,** 841–847

STERNBERGER L.A., HARDY P.H. JR, CUCULIS J.J. & MEYER H.G. (1970) The unlabelled antibody enzyme method of immunohistochemistry. Preparation and properties of soluble antigen–antibody complex (horse radish peroxidase anti-horseradish peroxidase) and its use in identification of spirochaetes. *Journal of Histochemistry and Cytochemistry* **18,** 315–333

SUN T.T., SHIH C. & GREEN H. (1979) Keratin cytoskeletons in epithelial cells of internal organs. *Proceedings of the National Academy of Sciences (USA)* **76,** 2812–2817

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Nephrology Dialysis Transplantation

Original Article

Distribution of IgE-Bearing Cells in Renal Biopsies in Wegener's Granuloma

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Abstract. A comparison was made between the renal biopsy appearances of Wegener's granuloma, microscopic polyarteritis and diffuse endocapillary proliferative glomerulonephritis, including the distribution of surface IgE-bearing mast cells. In microscopic polyarteritis and in other inflammatory conditions mast cells were usually associated with areas of interstitial inflammatory infiltrate, whereas in Wegener's granuloma mast cells were usually separate from areas of inflammation, suggesting a role for mast cells in the development of the renal lesion of Wegener's granulomatosis.

Key words: Renal Biopsies; Wegener's granuloma; IgE

Introduction

The differentiation between Wegener's granuloma and microscopic polyarteritis in renal biopsies is often impossible using standard techniques [1], since both classically show a focal and segmental necrotising vasculitis [2], whilst granulomata, the hallmark of Wegener's granuloma in nasal or lung biopsies, are hardly ever seen in renal biopsies [1]. The aetiology and pathogenesis of Wegener's granuloma is unclear, although some patients have a raised serum IgA [3] and others have raised serum IgE [4,5]. Circulating immune complexes are sometimes found [3,4,6], and some renal biopsies show deposition of immunoreactants within the glomeruli [7]. The partial success of antimicrobial therapy in Wegener's granuloma has led to the suggestion that host response to an underlying infection may trigger the disease [8]. Recently an IgG antineutrophil cytoplasm antibody has been found in the serum of patients with Wegener's granuloma [9] although this has not yet been shown to be of any pathogenetic significance. The inflammatory infiltrate in renal biopsies in Wegener's granuloma is predominantly T cell [10] and this has supported the hypothesis of increased hyperreactivity to inhaled antigens or pathogens [11,12]. Some authors have found that glomerular thrombosis and necrosis are the primary features of Wegener's granuloma [13]. Although immune complexes are not always found in glomeruli from patients with microscopic polyarteritis, there are well documented associations of microscopic polyarteritis with exogenous antigens, especially hepatitis B, and the vasculitis is thought to result from immunoglobulin deposition [2].

In view of the possible implications of the role of IgE and mast cells in Wegener's granuloma, we investigated a series of renal biopsies to find the distribution of deposited or cell-associated IgE and compared them with the findings in renal biopsies from patients with microscopic polyarteritis and diffuse endocapillary glomerulonephritis.

Materials and Methods

Patients were selected in whom only one of the two diagnoses had been established on standard clinical and histopathological criteria. They had systemic disease and presented at otolaryngology, renal, or general medical clinics. None of the patients had evidence of systemic lupus erythematosus or hepatitis B infection. Nine cases

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of Wegener's granuloma (seven male, two female; median age 49 years, range 19–63 years), and nine of microscopic polyarteritis (seven male, two female; median age 56 years, range 30–69 years) were included, as well as six cases of proliferative glomerulonephritis (four male, two female; median age 30 years, range 18–46 years. Initial renal function, as assessed by blood urea, was not significantly different between the Wegener's granuloma and microscopic polyarteritis groups (Wegener's granuloma urea 21.8 ± 6.4 mmol/l; microscopic polyarteritis were 21.9 ± 5.5 mmol/l). In addition, blocks from four normal kidneys were studied as controls.

Percutaneous renal biopsies were processed routinely as described previously [14] and in addition an assessment of the degree of inflammation (using a semiquantitative scale of 0–4) was made by two independent observers (DH, CN). A score of 0 was assigned when no inflammation was seen, 1 when there was minimal inflammation, 2 when a diffuse infiltrate or aggregates of inflammatory cells were seen, 3 when up to half the interstitium consisted of inflammatory cells, and 4 when areas of inflammation became confluent and were associated with severe tubular atrophy. The use of renal biopsies rather than nasal biopsies avoided the complication of non-specific inflammatory charge related to secondary infection.

Polyclonal rabbit anti-human IgE (Dako Ltd) was used in an immunoperoxidase system [14] to localise IgE. No IgE-containing immune complex deposits were identified, but two types of cell staining were seen, cytoplasmic staining, the cells being identified as plasma cells, and surface staining, indicating that the cells were mast cells. Because percutaneous biopsies were used there was insufficient tissue to permit an accurate quantitative assessment such as that used by Colvin and colleagues [16], so mast cells were scored according to whether or not the cells were solitary or associated with other inflammatory cells. The number of mast cells in each biopsy and the proportion of these cells associated with areas of inflammation was recorded. All renal biopsies studied renal cortex.

Results

General

The general histopathological features found were similar to those already described in the literature [1]. There was no evidence of IgE deposition in glomeruli, tubules, vessels or interstitium.

Cytoplasmic IgE Plasma Cells

A small number of IgE-plasma cells were seen in Wegener's granuloma, microscopic polyarteritis and proliferative glomuleronephritis biopsies (Fig. 1), and these were always associated with other inflammatory cells.

Biopsies were scored for inflammation by two blind observers on two separate occasions and results were never more divergent than one grade. The median scores were 2.0 (Wegener's granuloma), 2.0 (microscopic polyarteritis), 1.5 (proliferative glomuleronephritis) and 1.0 (control) respectively, and there was no significant difference between the Wegener's granuloma and microscopic polyarteritis groups.

Surface IgE-Bearing Mast Cells

Normal renal tissue contained very infrequent surface IgE-bearing mast cells in close proximity to peritubular capillaries and the vascular pole of glomeruli. In proliferative glomuleronephritis, Wegener's granuloma and microscopic polyarteritis there was an increased number of mast cells demonstrated by anti-IgE immunoperoxidase (Fig. 2), compared to controls, although the frequency of mast cells varied widely between biopsies (Table 1). It was also noted that the intensity of staining varied widely, even within the same biopsy. As expected, the proportion of mast cells associated with an inflammatory cell infiltrate increased as the degree of inflammation increased. However, at each grade of inflammation the proportion of mast cells found in association with inflammatory cells was lower in the Wegener's granuloma group than in the microscopic polyarteritis and proliferative glomerulonephritis groups (Table 1), indicating that in Wegener's granuloma, mast cells were more often unassociated with areas of inflammation. This difference between Wegener's granuloma and microscopic polyarteritis was statistically significant ($P < 0.02$, Mann–Whitney U Test, two-tailed) (Fig. 3).

Discussion

Mast cells identified by metachromatic stains or electron microscopy are found only rarely in the normal kidney, although their numbers increase in chronic inflammation and neoplasia [15,16]. In one study showing an increased number of mast cells in various inflammatory and vascular lesions, a quantitative assessment was made on biopsy and nephrectomy material [16]. In some of these cases vacuolated cells, thought to be degranulated mast cells, were seen actually within tubular epithelium. We have identified mast cells by their binding of IgE, and failed to see any in an intra-epithelial position.

The distribution of mast cells detected in our cases was similar for microscopic polyarteritis and proliferative glomerulonephritis, the majority of mast cells being present as part of a general inflammatory cell infiltrate. In Wegener's granuloma however, the pattern was different, the mast cells being identified predominantly without an associated inflammatory infiltrate, although still in the

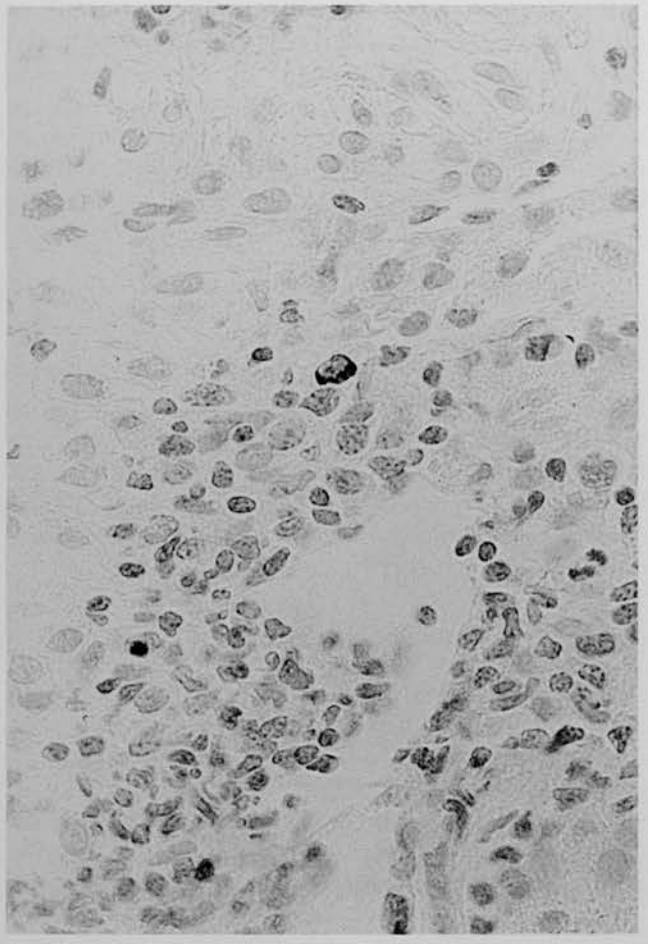

Fig. 1. Group of mixed inflammatory cells with one plasma cell showing cytoplasmic staining for IgE (Case of microscopic polyarteritis). × 225.

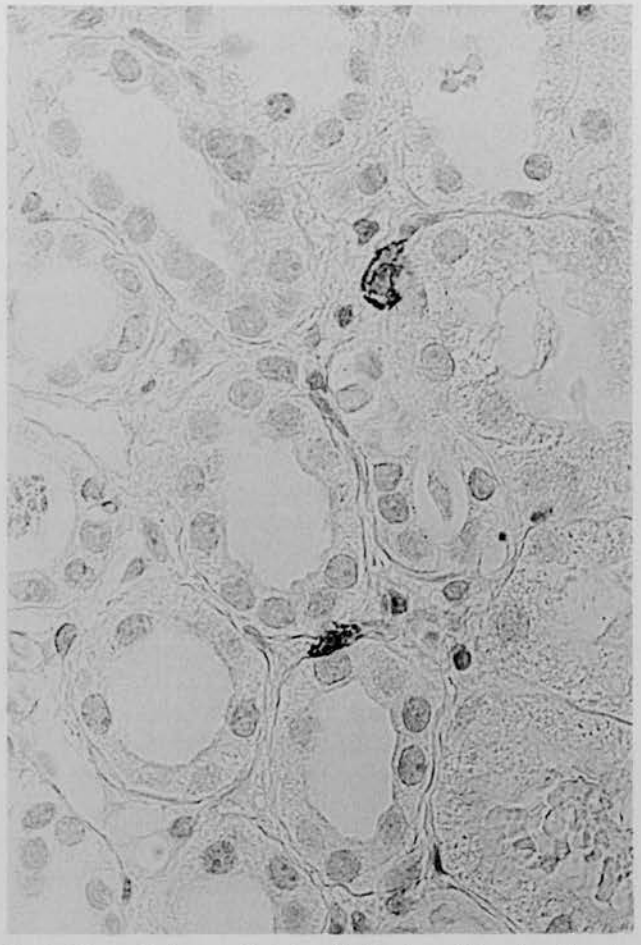

Fig. 2. Mast cells in a peritubular distribution with a rim of surface IgE stained by immunoperoxidase and unassociated with other inflammatory cells (Case of Wegener's granuloma). × 225.

typical perivascular sites seen in normals. This suggests that the increase in mast cells in Wegener's granuloma is a phenomenon preceding the inflammation, and may therefore be of some pathogenetic significance. It is unlikely that IgE immunoperoxidase demonstrates all mast cells, and the variability of staining intensity we have seen would support this view. There may be other mast cells present in the biopsies but not in an activated state, with insufficient bound IgE to be detectable by this technique.

On degranulation, induced by cross-linkage of surface-bound IgE, mast cells release a large number of mediators of inflammation and repair [17]. The association found between Wegener's granuloma and raised serum IgE in some patients [4,5] has led to the suggestion that mast cells in Wegener's granuloma may act in the development of a vasculitis by predisposing to increased vascular permeability and facilitating deposition of immune complexes in the vessel wall [18]. Recently, receptors for IgE have been described in some human mononuclear cells [19]. We have no evidence that IgE production by plasma cells in the kidney is increased in Wegener's granuloma, since comparable number of IgE plasma cells were identified in Wegener's granuloma, microscopic polyarteritis and proliferative glomerulonephritis. In Wegener's granuloma, compared with other non-allergic inflammatory nasal conditions, an increase in IgE-plasma cells in the nasal mucosa has been demonstrated (J Piris, personal communication). The serum IgE concentration was not measured in any of our patients at the time of renal biopsy.

We conclude that there is an increase in mast cells detectable by IgE immunoperoxidase in inflammatory conditions such as Wegener's granuloma, microscopic polyarteritis and proliferative glomerulonephritis, and that the distribution of mast cells in Wegener's granuloma is different. In these cases the release of mast-cell products may permit immune-complex deposition into vessel walls, with increased permeability, and so initiate the progression to vasculitis.

Acknowledgements. We are grateful to Dr A. C. Douglas and Dr M. K. MacDonald for permission to use clinical and pathological data on these patients.

Table 1. The number of mast cells present in renal biopsies and the proportion in areas of inflammation grouped according to the grade of inflammation and diagnosis

Grade of inflammation	WG	MPA	PGN	Control
	i/N (P)	i/N (P)	i/N (P)	i/N (P)
0	–	–	–	0/3 (0.00)
1	2/7 (0.29)	18/38 (0.47)	9/12 (0.75)	5/15 (0.33)
	7/42 (0.17)		0/1 (0.00)	1/3 (0.33)
	2/28 (0.07)		3/8 (0.38)	0/25 (0.00)
2	5/15 (0.33)	1/2 (0.50)	7/11 (0.64)	–
	16/74 (0.22)	5/7 (0.71)	11/25 (0.44)	
		6/10 (0.60)	7/12 (0.63)	
		2/2 (1.00)		
		1/3 (0.33)		
3	2/9 (0.22)	41/64 (0.64)	–	–
	31/66 (0.47)			
4	6/15 (0.40)	35/43 (0.81)	–	–
	54/96 (0.56)	19/24 (0.79)		

i, mast cells in inflamed areas; N, total number of mast cells; P, proportion of mast cells in inflamed areas; WG, Wegener's granuloma; MPA, microscopic polyarteritis; PGN, proliferative glomerulonephritis.

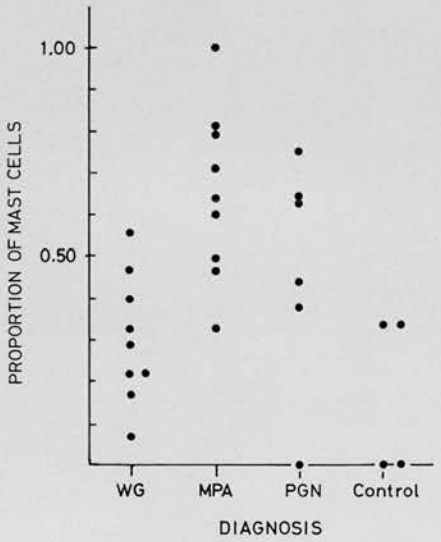

Fig. 3. Proportion of mast cells in areas of inflammation for each of the diagnostic groups. WG, Wegener's granuloma; MPA, microscopic polyarteritis; PGN, proliferative glomerulonephritis. The difference between the Wegener's granuloma and microscopic polyarteritis groups is significant ($P < 0.02$; Mann–Whitney).

References

1. Heptinstall RH. In: *Pathology of the Kidney* (3rd edit). Little, Brown, Boston, 1983: 793–838
2. Cupps TR, Fauci AS. In: *The Vasculitides*. W. B. Saunders, Philadelphia, 1981: 72–87
3. Fauci AS, Haynes BF, Katz P, Wolff SM. Wegener's granulomatosis: prospective clinical and therapeutic experience with 85 patients for 21 years. *Ann Intern Med* 1985; 98: 76–85
4. Conn DL, McDuffie FC, Holley KE, Shroeter AL. Immunologic mechanisms in systemic vasculitis. *Mayo Clinic Proc* 1976; 51: 511–518
5. Singh S, Yap PL, Douglas AC. Immunological parameters in midline granuloma. *J Clin Lab Immunol* 1982; 9: 71–74
6. Hu CH, O'Laughlin S, Winkelmann RK. Cutaneous manifestations of Wegener's granulomatosis. *Arch Dermatol* 1977; 113: 175–182
7. Fauci AS, Wolff SM. Wegener's granuloma and related diseases. In: Dowling HF ed. *Disease-a-Month*, vol. 23, Year Book Medical Publishers, Chicago, 1977: 1–36
8. DeRemee RA, McDonald TJ, Weiland LH. Wegener's granulomatosis: Observations on treatment with antimicrobial agents. *Mayo Clin Proc* 1985; 60: 27–32
9. van der Woude FJ, Lobatto S, Permin H et al. Autoantibodies against neutrophils and monocytes: tool for diagnosis and marker of disease activity in Wegener's granulomatosis. *Lancet* 1985; 1: 425–429
10. Berge IJ, Wilmink JM, Meyer CJ et al. Clinical and immunological follow-up of patients with severe renal disease in Wegener's granulomatosis. *Am J Nephrol* 1985; 5: 21–29
11. Godman GC, Churg J. Wegener's granulomatosis: pathology and review of the literature. *Arch Pathol* 1954; 58: 533–553
12. Douglas AC, Anderson TJ, MacDonald MK, Simpson JG. Midline and Wegener's granulomatosis. *Ann NY Acad Sci* 1976; 278: 618–635
13. Weiss MA, Grissman JD. Renal biopsy findings in Wegener's granulomatosis: segmental necrotising glomerulonephritis with glomerular thrombosis. *Hum Pathol* 1984; 15: 943–956
14. Harrison DJ, Thomson D, MacDonald MK. Membranous glomerulonephritis. *J Clin Path* 1986; 39: 167–171
15. Pavane-Macaluso M. Tissue mast cells in renal diseases. *Acta Pathol Microbiol Scand* 1960; 50: 337–346
16. Colvin RB, Dvorak AM, Dvorak HF. Mast cells in the cortical tubular epithelium and interstitium in human renal disease. *Hum Pathol* 1974; 5: 315–326
17. Schwartz LB, Austen KF. Structure and function of the chemical mediators of mast cells. *Prog Allergy* 1984; 34: 271–321
18. Leavitt RY, Fauci AS. Pulmonary vasculitis. *Am Rev Respir Dis* 1986; 134: 149–166
19. Spiegelberg HL. Structure and function of Fc receptors for IgE on lymphocytes, monocytes and macrophages. In: Kunkel HG, Dixon FJ eds. *Advances in Immunology*, Vol. 35, Academic Press, Florida, 1984: 61–88

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RENAL BIOPSY AND ANTINEUTROPHIL ANTIBODIES IN THE DIAGNOSIS AND ASSESSMENT OF WEGENER'S GRANULOMA

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Summary

Seventeen patients with Wegener's granuloma (WG), all of whom had renal biopsies taken at presentation, are reviewed. In conjunction with nasal or transbronchial biopsies renal biopsies aided diagnosis and also yielded prognostic information. The detection of antineutrophil cytoplasm antibodies in the serum of patients with WG is a useful supplementary diagnostic tool.

INTRODUCTION

The syndrome of classical Wegener's granuloma (WG) is a triad of necrotizing granulomatous vasculitis of lung and upper respiratory tracts and a focal and segmental necrotizing glomerulonephritis (1); its precise aetiology remains uncertain (2). Many patients have a raised serum IgA (3) and some have a raised serum IgE (4). Patients often present to otolaryngologists or respiratory physicians with non-specific signs and symptoms of a systemic inflammatory disease, as well as complaints of sinusitis, nasal ulceration and discharge, or haemoptysis (3). On some occasions renal failure may precipitate presentation (5) and up to 85% of cases at presentation will have renal involvement, albeit asymptomatic even in so-called limited WG (3). It is important to diagnose WG rapidly, since the untreated mortality is high, resulting in an average survival of only 5 months, renal failure being the commonest cause of death (6). Renal biopsy is sometimes performed but its value has been questioned because it is frequently non-specific (2), showing anything from minimal glomerular changes through focal and segmental glomerular lesions to crescentic glomerulonephritis with a small vessel vasculitis. Recently the detection of antineutrophil antibodies in the serum of patients with WG (7) has led to the hope of a specific serological test for WG, and its distinction from other forms of vasculitis. We present a series of 17 patients with WG, all of whom had renal biopsy at the time of presentation, and 10 of whom have had serum examined for the presence of antineutrophil antibodies.

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Materials and Methods

Seventeen patients were included in the study, in whom the diagnosis of WG was made on the basis of clinical assessment and histopathological findings. These patients had originally presented at otolaryngological, respiratory or renal clinics. A summary was made of clinical records. Percutaneous renal biopsies were taken, fixed in formalin and processed routinely (8). Antineutrophil cytoplasm antibodies were detected by an indirect immunofluorescence method (7) which involved the preparation of neutrophil-enriched cytospins, incubating with the patient's serum, and then with rabbit anti-human IgG-conjugated to fluorescein isothiocyanate label. One hundred and seventy control sera from other inflammatory conditions were also studied. These included microscopic polyarteritis, various forms of glomerulonephritis, Churg–Strauss syndrome, sarcoidosis and pneumonia.

RESULTS

Clinical features

These are summarized in Table I. There were 12 male and five female patients with a median age (range) at presentation of 45 years (19–68 years). The median (range) duration of symptoms before presentation was 6 months (1–60 months) and 15 of the 17 patients had upper and/or lower respiratory tract involvement as major presenting symptoms. In addition two cases had significant cutaneous involvement and in three cases severe renal failure was part of the initial symptom complex. Only two urine specimens from 15 patients examined microscopically were normal, many patients having haematuria and proteinuria. Eight of 14 presentation chest radiographs were abnormal, showing diffuse non-specific shadowing in five, consolidation of the right lower lobe in one, and cavitating lung lesions in two. Eight patients had normal blood urea at presentation (<7 mmol/litre) and nine had raised blood urea (>7 mmol/litre).

All patients received cytotoxic drug therapy and the 1-year survival was 80%. In 15 cases followed up for at least 1 year, five have died, one who had a normal blood urea at outset (one out of seven patients), and four who had a raised blood urea (four out of eight patients). Death was caused by septicaemia in three cases, the consequences of gastrointestinal haemorrhage and renal failure in one case, and pulmonary embolus in the context of active widespread vasculitis despite cytotoxic drug therapy in one case.

Pathological findings

Fourteen patients had nasal biopsies, and a firm or strongly suspected diagnosis of WG was made in seven of these because of the presence of a necrotizing vasculitis, multinucleate giant cells, granuloma formation and florid chronic inflammation. In the remaining biopsies no specific features suggestive of WG were identified. Two patients had transbronchial lung biopsies and one of these showed a necrotizing granulomatous vasculitis. Three of 17 renal biopsies showed a crescentic glomerulonephritis, five showed a focal and segmental necrotizing glomerulonephritis, four had a focal and segmental proliferative or sclerosing glomerular lesion, four had a diffuse mesangial proliferative glomerulonephritis, and only one was histologically normal. Nasal and renal biopsy findings are summarized in Table II.

When biopsies were scored for tubular atrophy on a 0–3 scale it was found that the

Table I. Summary of clinical data of 17 patients with WG

Patient	*Age* (years)	*Sex*	*Length of prodromal illness* (months)	*Initial urea* (mmol/litre)	*Presenting symptoms*	*Chest radiograph*	*Urine analysis*
1	23	M	3	50	Haemoptysis; URT*	Bilateral diffuse infiltrates	RBC casts; proteinuria
2	50	F	10	4	URT	Nad†	Nad
3	45	M	20	8	URT	Nad	RBC casts; proteinuria
4	39	M	5	3	URT; pyoderma	Nad	Haematuria; proteinuria
5	24	F	1	5	Arthritis; skin vasculitis	Nad	Not available
6	56	M	36	12	URT; epistaxis	Not available	Haematuria; granular casts
7	64	M	11	10	URT; epistaxis	Not available	Proteinuria; WBC and hyaline casts
8	31	M	3	9	Haemoptysis; cough	Right lower consolidation	Proteinuria; micro-haematuria
9	22	M	1	4	Haemoptysis; URT	Nad	Granular casts
10	43	M	6	30	URT; renal failure	Right basal shadowing	Proteinuria; WBC casts
11	54	F	24	5	URT	Diffuse increased markings	Nad
12	68	M	60	7	URT	Nad	RBC casts
13	57	F	5	30	URT	Right pleural effusions; atelectasis	Haematuria; RBC casts
14	39	M	6	5	Haemoptysis; URT	Not available	Haematuria
15	19	M	3	3	Epistaxis; URT; haematuria	Cavitating lesions right lung	Haematuria; proteinuria
16	63	M	1	22	Pyrexia; renal failure	Non-specific shadowing right base	Anuric
17	54	F	6	3	Haemoptysis; pleuritic pain	Cavitating lung lesions	Haematuria

*URT: upper respiratory tract lesion.
†Nad: no abnormality detected.

Table II. Summary of nasal and renal biopsy findings

Nasal/transbronchial	*Renal*	*Number*
Very suspicious	Segmental necrotizing	4
Very suspicious	Non-specific	4
Non-specific inflammation	Segmental necrotizing	3
Non-specific inflammation	Segmental proliferation	4
Non-specific inflammation	Non-specific	1

average score for biopsies from cases with normal blood urea at presentation was 0.9, compared with 1.9 for cases with raised blood urea. In several cases with low tubular atrophy scores but high blood urea, renal function improved on treatment (Fig. 1). In 80% of cases small amounts of immunoreactants were detected in glomeruli by immunofluorescence (IgM, IgA, IgG, C3) and 56% had varying amounts of hyalinization and fibrosis in

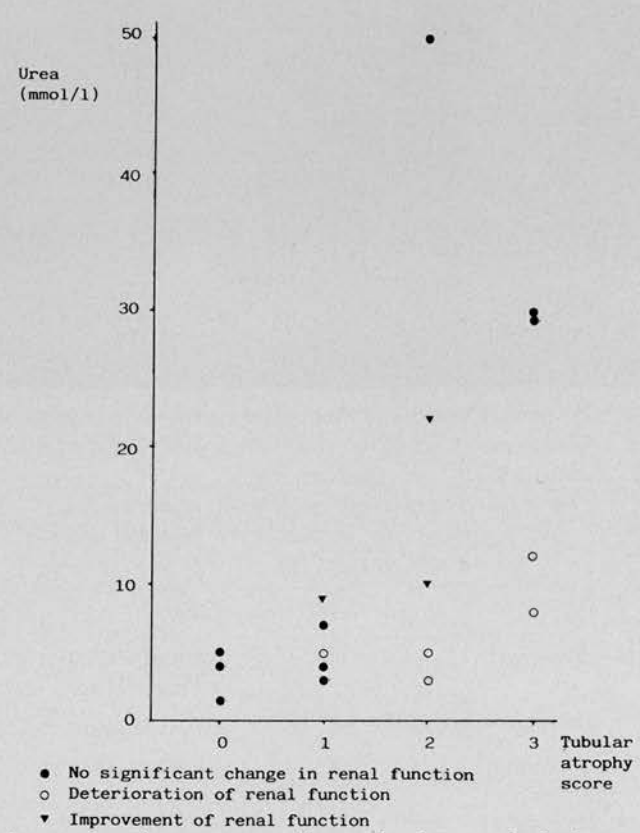

Fig. 1. This shows the presentation urea according to the degree of tubular atrophy scored on the renal biopsy from no significant atrophy (score 0) to severe tubular atrophy (score 3). Also recorded is the direction of change of the urea concentration on follow-up

the glomeruli or glomerula crescents. In only 13% was there no immunoreactive material. Electron microscopy showed no specific features.

Of the 10 sera examined by indirect immunofluorescence for the presence of IgG antineutrophil antibodies (Fig. 2), antibody was detected in nine at serum dilutions of at least 1:64, and in one case no antibodies were found. This negative case, however, had already begun therapy before the serum sample was taken. In two cases the antibodies disappeared with therapy and clinical improvement and in another two cases clinical relapse was associated with an increase in antibody titres. However, antineutrophil cytoplasm antibodies were also identified in some of the cases of microscopic polyarteritis and Churg–Strauss syndrome studied (Table III).

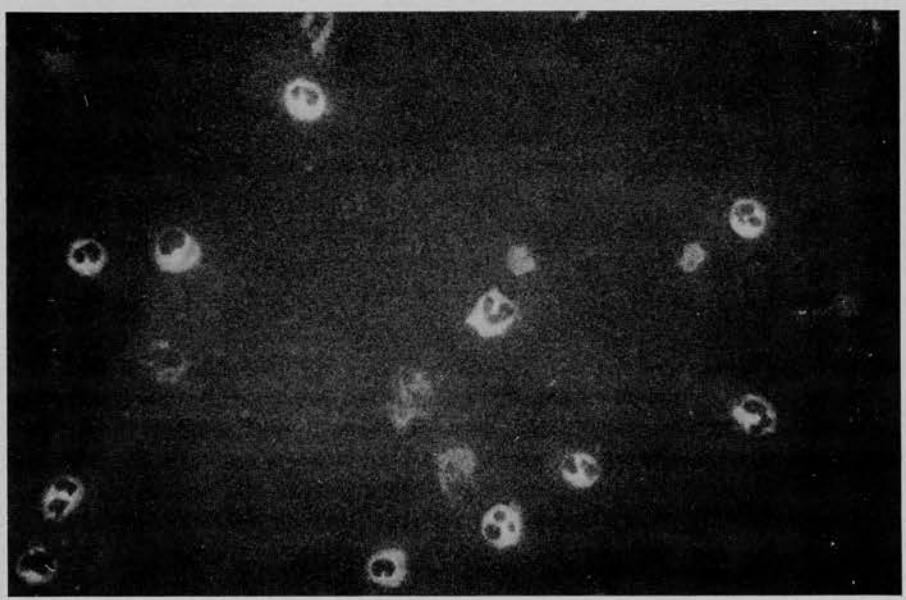

Fig. 2. Photomicrograph showing positive fluorescence of neutrophil cytoplasm after incubation with patient's sera at a dilution of 1:160 and then incubation with FITC conjugated anti-human IgG

Table III. Summary of results of study of antineutrophil antibodies in WG and other conditions

	WG	*Other*
Positive	9	2 Churg–Strauss 4 Polyarteritis
Weakly positive	0	3 Polyarteritis
Negative	1*	2 Polyarteritis* 138†

*On treatment.

†Includes glomerulonephritis, sarcoidosis, pneumonia, myocardial infarction.

DISCUSSION

Our clinical findings are in accord with many series in the literature in terms of age, sex ratio, clinical presentation, survival and cause of death (1). In a review of more than 100 cases of WG significant renal involvement was present clinically in more than 40% (9). Whilst only 50% of nasal and transbronchial biopsies were very suggestive of WG, the combination with the renal biopsy appearances would have suggested the possibility of WG, even though the appearances were not absolutely specific; severe chronic non-specific inflammation in a nasal biopsy combined with a focal and segmental glomerular lesion suggests a systemic process such as WG, even though a positive diagnosis of WG cannot be made. Unfortunately we have little information about renal function in patients with inflammatory nasal conditions who did not have WG, but it is most unlikely that they would show any renal impairment. Thus, in all but four cases, WG could be suggested on the combined appearances of nasal and renal biopsies. In the single case where the nasal biopsy showed non-specific changes and the renal biopsy showed mild diffuse mesangial proliferation, antineutrophil antibodies were detected at a serum dilution of 1:128. Although antibodies to neutrophil cytoplasm were thought to be specific for WG (7) it is now clear that in some other conditions these antibodies are also present, particularly microscopic polyarteritis (10) and Churg–Strauss syndrome (11). The presence of anti-neutrophil antibodies in our series is therefore not diagnostic of WG, but is still very suggestive in the presence of severe nasal inflammation.

The degree of renal tubular atrophy was a good indicator of the likely prognosis for renal function, even when the initial presentation of urea was misleading. This emphasizes the need for accurate assessment of the degree of permanent renal damage, with the institution of aggressive therapy as soon as possible. The degree of renal tubular atrophy has been regarded as a useful prognostic indicator in other renal diseases, such as membranous glomerulonephritis (12). The immunofluorescence findings do suggest a role for immune complexes in the pathogenesis of WG (13).

We conclude that renal biopsy has been shown to provide additional evidence to suggest the diagnosis of WG because it can demonstrate the systemic nature of the inflammatory process even if the classical features are not present. The degree of renal tubular atrophy may allow prediction of the likely degree of permanent renal impairment after initiation of therapy with cytotoxic drugs. Renal biopsy should therefore be considered as a useful investigation when confronted with cases of possible WG. The measurement of anti-neutrophil antibodies is a useful supplementary diagnostic technique for WG and may be a marker of disease activity (7, 14).

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REFERENCES

1. Cupps TR, Fauci AS. *Wegener's granulomatosis in the vasculitides*. Philadelphia: WB Saunders, 1981:72–87.
2. Leavitt RY, Fauci AS. Pulmonary vasculitis. *Am Rev Resp Dis* 1986;*134*:149–66.

3. Fauci AS, Hanes BF, Katz P, Wolff SM. Wegener's granulomatosis: prospective clinical and therapeutic experience with 85 patients for 21 years. *Ann Intern Med* 1983;*98*:76–85.
4. Singh S, Yap PL, Douglas AC. Immunological parameters in midline granuloma. *J Clin Lab Immunol* 1982;*9*:71–4.
5. Caralis PV, Falanga V, Pardo V. Delayed form of Wegener's granulomatosis. *S Med J* 1981;*74*:882–3.
6. Walton EN. Giant cell granuloma of the respiratory tract (Wegener's granulomatosis). *Br Med J* 1958;*2*:265–70.
7. van der Woude FJ, Rasmussen N, Lobatto S et al. Autoantibodies against neutrophils and monocytes: tool for diagnosis and marker of disease activity in Wegener's granulomatosis. *Lancet* 1985;*i*:425–9.
8. Harrison DJ, Thomson D, MacDonald MK. Membranous glomerulonephritis. *J Clin Path* 1986;*39*:167–71.
9. DeRemee RA, McDonald TJ, Weiland LH. Wegener's granulomatosis: experience with over 100 cases. New approaches for treatment. *Sarcoidosis* 1986;*3*:143–4.
10. Lockwood CM, Jones S, Moss DW, Bakes D, Whitaker KB, Savage COS. Association of alkaline phosphatase with an autoantigen recognised by circulating anti-neutrophil antibodies in systemic vasculitis. *Lancet* 1987;*i*:716–20.
11. Wathen CG, Harrison DJ. Circulating antineutrophil antibodies in systemic vasculitis. *Lancet* 1987;*i*:1037.
12. Stanziale P, Balletta M, Exposito A et al. Predictive factors of renal failure progression in membranous glomerulonephritis (MGN). *Kidney Int* 1985;*28*:259A.
13. Fauci AS, Wolff SM. Wegener's granulomatosis: studies in 18 patients and a review of the literature. *Medicine* 1973;*52*:535–61.
14. Gross WL, Ludemann G, Kiefer B, Beigel A, Niedermayer W, Lehmann H. Autoantibodies against neutrophils in Wegener's granulomatosis: immunodiagnostic significance. *Sarcoidosis* 1986;*3*:156.

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AUTOANTIBODIES TO NEUTROPHIL CYTOPLASMIC ANTIGENS IN SYSTEMIC VASCULITIS HAVE THE SAME TARGET SPECIFICITY

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SUMMARY

Many patients with systemic vasculitis have antibodies to neutrophil cytoplasm antigens (ANCA) detectable by indirect immunoflourescence. We sought to characterize further the nature of these antigens. Western blots of neutrophil protein extracts indicated that nine patients' sera, positive for ANCA by immunofluorescence, all reacted with a 45 kDa and a 27–31 kDa protein. Negative control sera, and sera taken in remission, did not react with either of these antigens. The results suggest that ANCA in vasculitis have the same target specificity and may therefore permit greater accuracy of diagnosis and increase our understanding of the pathogenesis of the conditions.

KEY WORDS—Vasculitis, neutrophil, autoantibodies, Western blotting.

INTRODUCTION

Wegener's granulomatosis is an uncommon systemic vasculitis characterized by a necrotizing granulomatous vasculitis of lower and upper respiratory tracts, and a focal and segmental necrotizing glomerulonephritis.[1] However, any organ can be involved and the diagnosis may be difficult to make and hence therapy is delayed.[2] In 1985, Van der Woude *et al.*[3] described the presence of IgG antibodies in the serum of patients with Wegener's granulomatosis which were directed against a component of neutrophil cytoplasm. Initially antibodies to neutrophil cytoplasm antigens (ANCA) were thought to be specific for Wegener's,[3,4] but it is now clear that ANCA are present in some patients with microscopic polyarteritis[5–8] and Churg–Strauss syndrome.[7] The titre of antibody also appears to be a monitor of disease activity[1,8–10] Classically described ANCA give bright, coarsely granular, cytoplasmic fluorescence when incubated with cytospins of normal neutrophils,[1] but other types of ANCA giving weaker, more diffuse fluorescence are also being recognized in many other conditions,[11] including primary biliary cirrhosis,[12] Paget's disease of bone (upublished observations), and rheumatoid arthritis.[8] Since the pattern of fluorescence of many autoantibodies is difficult to reproduce reliably,[13] it is desirable to know the nature of the antigen recognized by ANCA in vasculitis, so that more specific assays can be developed. This study aimed to characterize further the autoantibodies in systemic vasculitis by the extraction of neutrophil proteins, separation by polyacrylamide gel electrophoresis, and immunoblotting.

MATERIALS AND METHODS

Patients

Serum from seven patients with biopsy-proven Wegener's granulomatosis (nasal, lung, renal) and two with microscopic polyarteritis (renal) were selected. None of the patients had received immunosuppressive therapy and all had evidence of active systemic disease. Five of the patients were male and the mean age was 49 years (range 31–70). Eight

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control samples from healthy volunteers were also studied, five of whom were male; mean age 39 years (range 22–70). Two further samples from patients with glomerulonephritis who had weak anti-neutrophil cytoplasm antibody activity were studied.

Indirect immunofluorescence

This was essentially the same method as described by Van der Woude *et al.*[3] Briefly, neutrophils were prepared from the peripheral blood of a healthy volunteer by dextran sedimentation[14] and neutrophil cytospins were made. Patient or control serum was added at dilutions of at least 1:20 for 30 min at room temperature. After washing, fluorescein-conjugated sheep anti-human IgG was added (1:50; SAPU, U.K.) for 30 min. Slides were washed, mounted in glycerol saline, and examined under ultraviolet light.

Protein extraction

Three fractions were obtained: water-soluble proteins by repeat freeze–thawing of the neutrophil pellet; urea-soluble proteins by incubation in 50 mM Tris buffer containing 8 M urea for 2 h at 25°C; and sodium dodecyl sulphate (SDS)- soluble proteins by incubation in Tris buffer containing 1 per cent SDS for 3 h at 37°C.[15]

All extractions were performed in the presence of mercaptoethanol and protease inhibitors, and extracts were snap-frozen in liquid nitrogen for storage prior to use.

Polyacrylamide gel electrophoresis

Proteins were separated according to molecular weight by running in an SDS-polyacrylamide gel. Gels contained 15 per cent polyacrylamide and were prepared by standard methods.[16] Each gel lane was loaded with 30 μg of neutrophil protein extract in SDS-loading buffer. In each gel, one lane contained a mixture of proteins of known molecular weight conjugated to coloured dyes (Rainbow Marker, Amersham, U.K.) from which the molecular weights of neutrophil proteins could be calculated. Proteins were transferred from gels to nitrocellulose paper (Hybond C, Amersham, U.K.) by electrophoresis.[17]

Immunodetection

Non-specific protein binding sites on the nitrocellulose paper were blocked by washing for 3 h in TBS containing 3 per cent bovine serum albumin (BSA) (BDH, U.K.) and 2 per cent reconstituted dried milk (Cadbury, U.K.) Filters were cut into strips corresponding to gel lanes and incubated with serum at dilutions of 1:100 to 1:1000 in 0·1 per cent Tween 20, 0·25 per cent BSA, 5 per cent goat serum (SAPU, U.K.)/TBS for 1 h. After washing, biotinylated goat anti-human IgG (1:1000; Amersham, U.K.) was added. Detection of bound antibody was with the Blu-Gene streptavidin/alkaline phosphatase kit (Gibco, BRL Ltd., U.K.) and bromochloroindolyl phosphate/Nitro blue tetrazolium, BCIP/NBT (Gibco Ltd.) (TBS, pH 9·5, containing 0·38 M BCIP, 0·4 M NBT, 10 mM $MgCl_2$) was used as the substrate. The reaction was stopped after 5 min by washing filters in a large excess of 'stop buffer' (20 mM Tris–HCl, pH 7·5, 0·5 mM Na_2 EDTA) so that positive bands were seen as purple on a white background. Control conditions (omission of patient serum and/or goat anti-human antibody) confirmed the absence of non-specific binding).

Results were obtained by comparing blot preparations and by scanning laser densitometry using a Chromoscan III Densitometer (Joyce-Loebl, F.R.G.) which records the position and intensity of bands in graphical form.[18] This had the advantage of allowing shorter incubation time with substrate which resulted in very faint bands by eye, but very low backgrounds by laser densitometry.

RESULTS

Immunofluorescence

All patients had ANCA which gave coarse granular cytoplasmic fluorescence by indirect immunofluorescence at serum dilutions of at least 1:80. No control sera gave any fluorescence at dilutions of 1:20 or greater.

Immunoblotting

Water-soluble extract—At serum dilutions of 1:100, both ANCA positive and negative sera gave a large number of bands. As serum was diluted, the number of bands reduced to zero at 1:1000. At no dilution were consistent differences found between ANCA positive and negative sera (Fig. 1). One patient with ANCA and microscopic polyarteritis had antibodies which reacted with a 21 kDa protein (Fig. 2). This did not correlate with any known clinical or laboratory parameter.

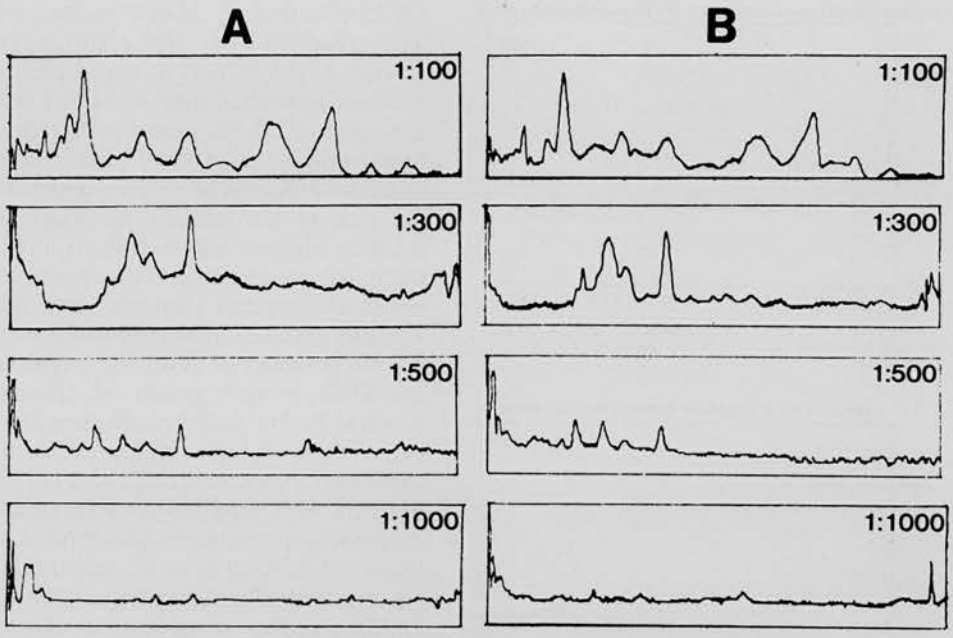

Fig. 1—Laser densitometrograms demonstrating the density of bands (vertical axis) against decreasing molecular weight (horizontal axis) for serial dilutions of an ANCA positive (A) and negative (B) serum reacted with water-soluble protein extract. Note the occurrence of many bands at higher concentrations of serum representing natural autoantibodies

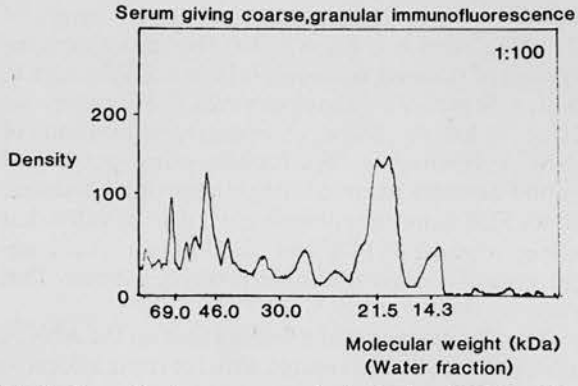

Fig. 2—Laser densitometrogram obtained using ANCA positive serum from a patient with microscopic polyarteritis which reacted with a protein at around 21 kDa in the water-soluble protein extract.

Urea-soluble extract—At low dilutions, numerous bands were recognized but serial dilution showed no consistent difference between ANCA positive and negative sera.

SDS-soluble extract—While at serum dilutions of 1:100 both groups of serum reacted with numerous bands, at dilutions of 1:500 only ANCA positive sera reacted with a 45 kDa and a 27–31 kDa protein (Fig. 3). This was consistent for all the sera studied (Fig. 4) and was reproducible. Sera from patients with inactive disease and no detectable ANCA also failed to recognize these bands. At serum dilution of 1:1000, some ANCA positive sera continued to recognize the 27–31 kDa protein. Six samples from patients with active sarcoidosis (2), active tuberculosis (2) and systemic lupus (2), which were ANCA negative, did not give bands at these molecular weights.

DISCUSSION

Since the description of ANCA in 1985, many groups have found them to be of value in the diagnosis of systemic vasculitis, in particular Wegener's granulomatosis.[3–7,9] The indirect immunofluorescence assay has a specificity of around 85 per cent

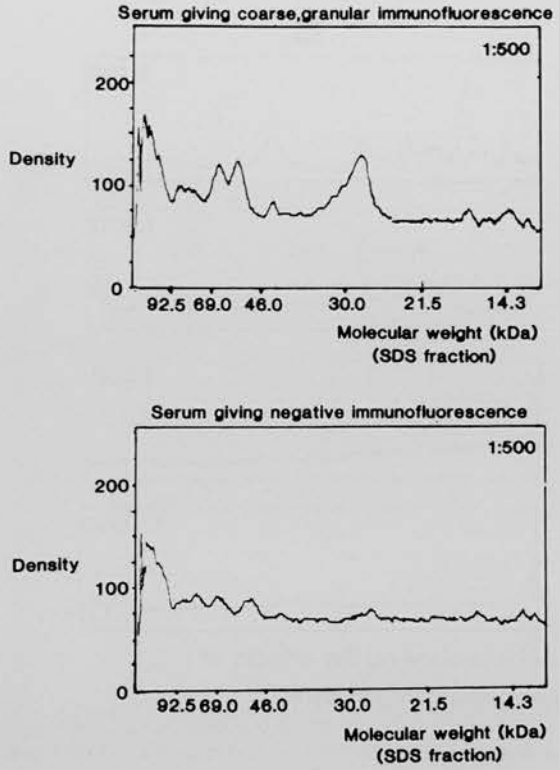

Fig. 3—At serum dilution of 1:500 an ANCA positive serum (top) reacts with two proteins at 45 and 27–31 kDa in the SDS-soluble fraction, which negative serum (bottom) does not recognize

and a sensitivity of about 75 per cent. However, there are problems in ensuring that separate laboratories agree to the criteria used for a particular immunofluorescence pattern to be pathognomonic of vasculitis,[11] and this is not always easy.[13] The identification of particular antigens would permit standardization of ANCA detection techniques and also allow more reliable quantification of ANCA.

Naturally occurring autoantibodies, IgM, and also IgG, to a variety of self antigens are well described in normal human serum employing a variety of techniques[19,20] and autoantibodies constitute a substantial part of normal circulating immunoglobulins.[19] Pathological autoantibodies with specific disease association therefore may differ from natural autoantibodies either in the antigen recognized, or in the titre of antibody, or both.[18,21] This does not necessarily imply a role in the pathogenesis of the associated disease.[19,21–23]

We have confirmed that both control and patient sera contain natural autoantibodies, and have shown that ANCA positive sera recognize epitopes on 45 kDa and 27–31 kDa proteins. Lockwood *et al.*[5] suggested that the epitopes recognized by ANCA might be part of neutrophil alkaline phosphatase. However, their work has been extensively criticized.[11,24,25] They used highly degraded hydrolysed samples of protein[24] and radioimmunoassay at serum dilutions of only 1:4 which would increase the risk of non-specific binding.[25] Furthermore, alkaline phosphatase in human unlike rabbit neutrophils is present diffusely in the cell membrane and not in cytoplasmic granules[26] and therefore would not give granular immunofluorescence.[25]

The presence of ANCA-recognized antigens in the SDS fraction would be consistent with the proteins being membrane-associated rather than cytoskeletal (urea-soluble) or cytosolic (water-soluble).[15] The absence of these bands in sera from patients with sarcoidosis, tuberculosis, and lupus erythematosus indicates that ANCA sera react with these antigens specifically and not just as part of reactive polyclonal hyperplasia in active disease. Goldschmeding *et al.*[27] have also reported that ANCA react with a protein of molecular weight 27–29 kDa which they suggest is present in azurophilic (primary) granules. They did not describe a minor antigen at 45 kDa but this may be because they did not perform a differential extract of protein based on solubility but prepared a whole cell extract in SDS. Smaller bands may therefore have been obscured.

Of the proteins present in human neutrophil azurophilic granules elastase has a molecular weight of 29 kDa,[28] and it is known that the serum concentration of released neutrophil elastase is very high in active Wegener's granulomatosis.[10] However, we have failed to show competitive inhibition of ANCA binding to neutrophils using polyclonal rabbit antisera against human neutrophil elastase. Since SDS denatures proteins, it is also possible that the epitopes at 45 kDa and 27–31 kDa are subcomponents of a larger molecule, possibly a dimer. This requires further study.

The significance of the finding that all the ANCA positive sera studied reacted with the same antigenic targets is great. The way is now open to isolate the protein(s), identify them, and use them in an enzyme linked immunoabsorbent assay (ELISA) to improve the diagnostic accuracy and to assess the value of monitoring ANCA as a marker of disease.

If ANCA do have a pathogenetic role in vasculitis, then the identification of target antigens may allow, as has been suggested by others,[5] specific forms of therapy based on plasma exchange and

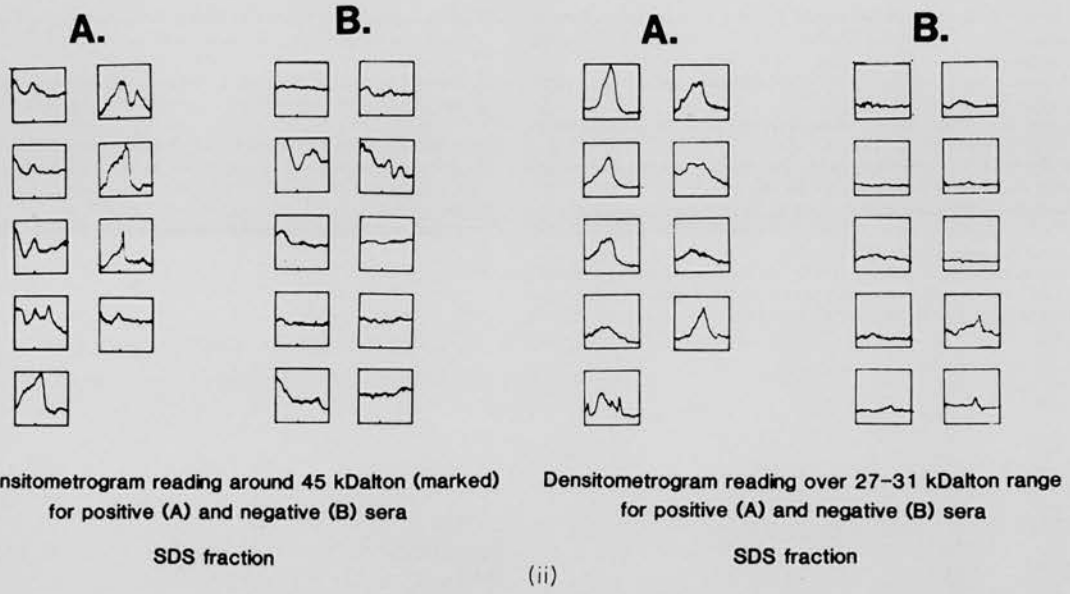

Fig. 4—Summary of results showing ANCA positive (A) and negative (B) sera at 1:500 reacted with SDS-soluble fraction. ANCA positive sera recognize bands at (i) 45 kDa and (ii) 27–31 kDa

selective immunoabsorption of the pathogenic autoantibodies.

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REFERENCES

1. Cupps TR, Fauci AS. Wegener's granulomatosis. In: The Vasculitides. New York: E.B. Saunders, 1981; 26–87.
2. Leavitt RY, Fauci AS. Pulmonary vasculitis. *Am Rev Respir Dis* 1986; **134:** 149–166.
3. Van der Woude FJ, Rasmussen N, Lobatto S, *et al.* Autoantibodies against neutrophils and monocytes: Tools for diagnosis and marker of disease activity in Wegener's granulomatosis. *Lancet* 1985; **1:** 425–429.
4. Gross WL, Ludemann G, Kiefer G, Lekmann H. Anticytoplasmic antibodies in Wegner's granulomatosis. *Lancet* 1986; **1:** 806.
5. Lockwood CM, Bakes D, Jones S, Whitaker KB, Moss DW, Savage COS. Association of alkaline phosphatase with an autoantigen recognised by circulating antineutrophil antibodies in systemic vascultis. *Lancet* 1987; **1:** 716–720.
6. Venning MC, Arfeen S, Bird AG. Antibodies to neutrophil cytoplasmic antigen in systemic vasculitis. *Lancet* 1987; **2:** 850.
7. Harrison DJ, Simpson R, Neary C, Wathen CG. Renal biopsy and antineutrophil antibodies in the diagnosis and monitoring of Wegener's granulomatosis. *Br J Dis Chest* 1988; **82:** 398–404.
8. Savage COS, Jones S, Winears CG, Marshall PD, Lockwood CM. Prospective study of radioimmunoassay for antibodies against neutrophil cytoplasm in the diagnosis of systemic vasculitis. *Lancet* 1987; **1:** 1389–1393.
9. Ludemann G, Gross WL. Autoantibodies against cytoplasmic structures of neutrophil granulocytes in Wegener's granulomatosis. *Clin Exp Immunol* 1987; **69:** 350–357.
10. Wathen CG, Bell D, Harrison DJ, Jackson M, Davies J. Serum human neutrophil elastase is increased in patients with active Wegener's granulomatosis. *Thorax* 1987; **42:** 750.
11. Rasmussen N, Wilk A, Hoier-Madsen M, Borregaard N, Van der Woude FJ. Antineutrophil cytoplasm antibodies. *Lancet* 1988; **1:** 706–707.
12. Gross WL, Ludemann G, Schroder J-M. Antineutrophil cytoplasm antibodies in Wegener's granulomatosis are not directed against alkaline phosphatase. *Lancet* 1987; **1:** 1488–1489.
13. Dighiero G, Magnac J, De Saint-Martin N, Avrameas S. Detection of antimitochondrial antibodies by ELISA and Western blot techniques and identification by one and two dimensional gel electrophoresis of M2 target antigens. *Clin Exp Immunol* 1987; **70:** 640–648.
14. Segal AW, Peters TJ. Analytic subcellular fractionation of human granulocytes with special reference to localisation of enzymes involved in microbicidal mechanisms. *Clin Sci Mol* 1977; **52:** 429–442.
15. Patek CE, Vornhagen R, Rink N, Clayton RM. Developmental changes in membrane protein expression by chick lens cells *in vivo* and *in vitro* and the detection of main intrinsic polypeptide (MIP). *Exp Eye Res* 1986; **43:** 29–40.
16. Tijsson TE. Localisation and identification of electrophoretically separated antigens after their transfer from gels to membranes. In: Burdon BH, Van Kippenberg PH, eds. Practise and Theory of Enzyme Immunoassays. Amsterdam: Elsevier, 1987; 423–448.
17. Bittner M, Kupferer P, Morris CF. Electrophoretic transfer of proteins and nucleic acids from slab gels to diazobenzyloxymethyl cellulose or nitrocellulose sheets. *Anal Biochem* 1986; **102:** 459–471.
18. Kyriatsoulis A, Manns M, Gerken G, *et al.* Distinction between natural and pathological auto-antibodies by immunoblotting and densitometric subtraction: liver–kidney microsomal antibody (LKM) positive sera identify multiple antigens in human liver disease. *Clin Exp Immunol* 1987; **70:** 53–60.

19. Dighiero G, Lymberi P, Guilbert B, Ternyck T, Avrameas S. Natural autoantibodies constitute a substantial part of normal circulating immunoglobulins. *Ann NY Acad Sci* 1986; **475:** 135–145.
20. Holmberg D, Coutinho A. Natural antibodies and autoimmunity. Immunol Today 1985; **6:** 356–357.
21. Cruse JM, Lewis RE. Contemporary concepts of autoimmunity. *Concepts Immunopathol* 1985; **1:** 1–31.
22. Walport M. The identification of autoantigens, autoantibodies, and their idiotypes. *Br J Rheum* 1987; **26:** 161–167.
23. Bach JF, Jacob L, Feutren G, Tron F. The questionable role of anti-DNA antibodies in the pathogenesis of systemic lupus erythematosus. *Ann NY Acad Sci* 1986; **475:** 231–240.
24. Goldschemeding R, Tetteroo PAT, Von der Borne AEG , Kallenberg CGM. Antineutrophil cytoplasm antibodies in Wegener's granulomatosis are not directed against alkaline phosphatase. *Lancet* 1987; **1:** 1489.
25. Rasmussen N, Borregaard N, Wiik A. Antineutrophil cytoplasm antibodies in Wegener's granulomatosis are not directed against alkaline phosphatase. *Lancet* 1987; **1:** 1488.
26. Dewald B, Bretz V, Baggiolini M. Release of gelatinase from a novel secretory compartment of human neutrophils. *J Clin Invest* 1982; **70:** 518–525.
27. Goldschmeding R, Tetteroo PAT, Von der Borne AEG. Wegener's granulomatosis specific autoantibodies (ACPA). Characterisation of the antigen. *Kidney Int* 1987; **32:** 779.
28. Baugh RJ, Travis J. Human leukocyte granule elastase. Rapid isolation and characterisation. *Biochemistry* 1976; **15:** 836–841.

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Antibodies to neutrophil cytoplasmic antigens in Wegener's granulomatosis and other conditions

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Antibodies to neutrophil cytoplasmic antigens in Wegener's granulomatosis and other conditions

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ABSTRACT The use of serum antibodies to neutrophil cytoplasmic antigens (ANCA) as a diagnostic marker for Wegener's granulomatosis and other forms of vasculitis has been assessed. Although ANCA have been described by several groups the precise antigenic targets are unknown, and detection of ANCA still relies on an indirect immunofluorescence assay technique. Several different patterns of fluorescence have been produced by using sera from different groups of patients, and insufficient information is available on the frequency of positive results and of the patterns of immunofluorescence obtained when serum from patients with vasculitis as a part of a generalised connective tissue disease is used. A study was carried out on serum from 240 patients, including 23 patients with Wegener's granulomatosis, 12 with microscopic polyarteritis, and 30 with various connective tissue diseases. Three patterns of fluorescence were observed: bright coarsely granular cytoplasmic, bright non-granular cytoplasmic, and weak diffuse cytoplasmic. The bright, coarsely granular pattern was 86% specific for Wegener's granulomatosis in this series and was observed in 18 of 23 cases. Other patterns of fluorescence were found in various conditions and were not of diagnostic value. The technique is simple, inexpensive, rapid, and reproducible.

Introduction

Wegener's granulomatosis is classically defined as a necrotising granulomatous vasculitis in the upper and lower respiratory tracts associated with focal and segmental necrotising glomerulonephritis.[1] The classical features are frequently absent, however, and diagnosis is therefore delayed.

The recognition of autoantibodies directed against a neutrophil cytoplasmic antigen (ANCA) in Wegener's granulomatosis[2 3] has led to hopes of a specific diagnostic marker for this disease. ANCA may also be useful in monitoring disease activity,[2 3] particularly when used with measurement of C-reactive protein.[4]

It is clear that ANCA represent a group of different antibodies as different patterns of fluorescence may be obtained from different serum samples.[5] The original description described bright, granular fluorescence of the neutrophil cytoplasm in an indirect immunofluorescence assay as diagnostic of Wegener's granulomatosis.[2] ANCA have, however, been described in microscopic polyarteritis,[6 7] Kawasaki disease,[8] Churg-Strauss syndrome,[9] and even carcinoma of the lung.[10] In microscopic polyarteritis, where the pattern of fluorescence has been specified,[6 7] a bright, diffuse cytoplasmic fluorescence has been recorded.

This study investigates the pattern of fluorescence produced by ANCA in serum samples from patients with a wide range of diseases, and assesses the clinical value of detecting ANCA by an indirect immunofluorescence assay.

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Methods

IMMUNOFLUORESCENCE ASSAY

The assay used is a modification of that described by Van der Woude and colleagues.[2] In brief, heparinised venous blood from a healthy volunteer was mixed with half its volume of 5% Dextran 250 (Pharmacia, UK) in 0·9% sodium chloride solution, and incubated at 37°C for 40 minutes to sediment red blood cells. The neutrophil enriched supernatant was washed twice in

phosphate buffered saline and resuspended at a cell concentration of 5×10^5 cells/ml. Aliquots of 100 μl were used to make cytospin preparations, which were fixed in absolute ethanol at 4°C for five minutes. In some experiments cytospin preparations were fixed in acetone at 4°C for five minutes. They were used either immediately or within five weeks, having been wrapped and stored at −20°C until use.

Cytospin preparations were incubated with the patient's serum serially diluted from 1:20 or 1:80 in phosphate buffered saline for 45 minutes. After two washes in the saline they were incubated with a 1:50 dilution of fluorescein conjugated rabbit antihuman IgG (Scottish Antibody Production Unit, Carluke) for 30 minutes before examination with an ultraviolet microscope. A known positive and a known negative serum sample were included each time the assay was performed.

Cytospin preparations were scored according to the nature and brightness of fluorescence to give four groups: 1—no appreciable fluorescence; 2—weak, diffuse cytoplasmic fluorescence; 3—bright but not coarsely granular cytoplasmic fluorescence; 4—bright, coarsely granular cytoplasmic fluorescence identical to or brighter than the positive control. The presence of antinuclear antibodies was also recorded. The dilution of serum at which fluorescence disappeared was also recorded for positive cases.

CLINICAL DIAGNOSES

The diagnosis was recorded for each patient studied without prior knowledge of the presence or absence of ANCA. The diagnosis of Wegener's granulomatosis was supported in every case by the histological appearance of biopsy material obtained from at least one affected tissue. Diagnoses were not altered in the light of the ANCA findings.

Results

CLINICAL

Twenty three patients with Wegener's granulomatosis were studied. A brief summary of symptoms at presentation and biopsy findings is given in table 1. Serum samples from a further 217 patients were also studied, including 12 patients with microscopic polyarteritis, three with Churg-Strauss syndrome, 38 with various connective tissue diseases, 41 with renal disease, 10 with malignancy, and the remainder with various inflammatory and infectious disorders (table 2).

IMMUNOFLUORESCENCE

No normal volunteers had ANCA detectable at serum dilutions of 1:20 or greater. Results of the ANCA test are summarised in table 2. The scoring system for ethanol fixed cytospin preparations was found to be reliable and reproducible, and two pathologists not concerned with the project concurred with the scores we had assigned. The scoring was helped by the inclusion of a known positive sample in each assay. When acetone was used as a fixative the pattern of fluorescence was usually diffuse and only erratic granular staining was seen. Serum samples from 21 patients, of whom 18 had Wegener's granulomatosis, showed the very bright, coarsely granular cytoplasmic fluorescence characteristic of the positive control (fig 1).

There were 23 cases of Wegener's granulomatosis in total, giving a sensitivity of 78% for the test. Of the 217 patients studied who did not have Wegener's granulomatosis, only three had a positive result. This gives a specificity of 86%, though this would be influenced by the composition of the control group. Nineteen patients, of whom two had Wegener's granulomatosis, had bright but non-granular cytoplasmic fluorescence (fig 2).

As the fluorescence score decreased the range of diseases with detectable ANCA increased. The inclusion of patterns other than the very bright

Table 1 *Details of patients with Wegener's granulomatosis*

Age (y)	*Sex*	*Presentation*	*Biopsy**	*ANCA pattern*
23	M	Haemoptysis	Nasal, renal	Coarse granular
40	M	Haemoptysis	Nasal	,,
42	F	Haemoptysis	Nasal	,,
53	F	Mouth or nose ulcers	Nasal, (renal)	,,
45	M	Renal failure	Nasal, renal	,,
39	M	Pyoderma, epistaxis	Nasal, (renal)	,,
24	F	Skin vasculitis	(Nasal), (renal)	,,
64	M	Epistaxis	(Nasal), renal	,,
30	M	Sinusitis	Nasal, renal	,,
31	M	Haemoptysis	Nasal, renal	,,
39	M	Renal failure	Nasal, (lung), renal	,,
38	M	Renal failure	Nasal, renal	,,
38	M	Haemoptysis	Lung	,,
63	M	Fever, lung consolidation	Renal	,,
57	F	Haemoptysis, haematuria	(Nasal), (renal)	,,
54	F	Haemoptysis	Transbronchial, renal	,,
39	M	Haematuria	(Nasal), renal	,,
70	F	Epistaxis, renal failure	(Nasal)	,,
43	M	Renal failure	(Nasal), renal	Non-granular
26	F	Cavitating lung lesions	Lung	,,
26	F	Epistaxis	Nasal, transbronchial	Weak, diffuse
28	F	Epistaxis	Nasal	
43	M	Nasal ulceration	Nasal, (renal)	Nil

*Parenthesis indicates that although the biopsy was performed it yielded findings that did not support the diagnosis of Wegener's granulomatosis. In other cases biopsy findings were either consistent with or diagnostic of systemic vasculitis.
ANCA—antineutrophil cytoplasmic antibodies.

Table 2 *Results of immunofluorescence findings for antineutrophil cytoplasmic antibodies (ANCA) according to the nature of the cytoplasmic fluorescence and the clinical diagnosis*

Cytoplasmic fluorescence	*No of cases*	*Diagnosis (n)*
Bright coarsely granular	21	Wegener's granulomatosis (18) Microscopic polyarteritis (2)* Churg-Strauss syndrome (1)†
Bright, non-granular	19	Wegener's granulomatosis (2) Microscopic polyarteritis (3) Mixed connective tissue disease (3) Paget's disease of bone (1) Churg-Strauss syndrome (1) Behçet's syndrome (1) Wegener's granulomatosis (treated) (2) Nephrosclerosis (2) Polymyalgia rheumatica
Weak	38	Wegener's granulomatosis (2) Microscopic polyarteritis (5) Mixed connective tissue disease (2) Rheumatoid arthritis (3) Churg-Strauss syndrome (1) Paget's disease of bone (3) Other diagnoses (22)‡
Insignificant	162	Wegener's granulomatosis (1) Microscopic polyarteritis (2) Other diagnoses, including pneumonia, nasal polyps, myeloma, Behçet's syndrome, sarcoidosis, Goodpasture's syndrome, polymyalgia rheumatica, Wegener's granulomatosis (treated), polyarteritis (treated)

*Focal and segmental necrotising glomerulonephritis.
†Eosinophilia, transbronchial biopsy.
‡Including Wegener's granulomatosis and polyarteritis during treatment, glomerulonephritis, sarcoidosis, systemic lupus erythematosus, Goodpasture's syndrome.

granular cytoplasmic fluorescence therefore resulted in reduced specificity of the assay.

ANCA were readily detectable in neutrophil cytospin preparations stored for up to five weeks. This allows the preparation of large batches of fixed slides, which is convenient if the test is to be performed rapidly as a diagnostic aid when required. Repeated freeze-thawing of serum samples resulted in a reduction in ANCA fluorescence, but samples stored for four years at −70°C retained fluorescence.

As serum giving a coarsely granular pattern was diluted we noted that the pattern of fluorescence changed to diffuse before the fluorescence signal actually disappeared. This change occurred at a serum dilution of 1:80 in one case, at 1:160 in most cases, and at 1:500 in only two cases. Repeat samples from patients having treatment sometimes showed a similar change in pattern of fluorescence from granular to diffuse, irrespective of the titre of antibody used.

The titre of antibody fell in parallel with other markers of disease activity, such as the white blood cell count, erythrocyte sedimentation rate, and serum concentration of human neutrophil elastase.[11] With recrudescence of clinical disease, such as mouth ulceration, lung cavitation, or deteriorating renal function, the titre of ANCA increased.

Discussion

The classification of vasculitis is difficult and at times

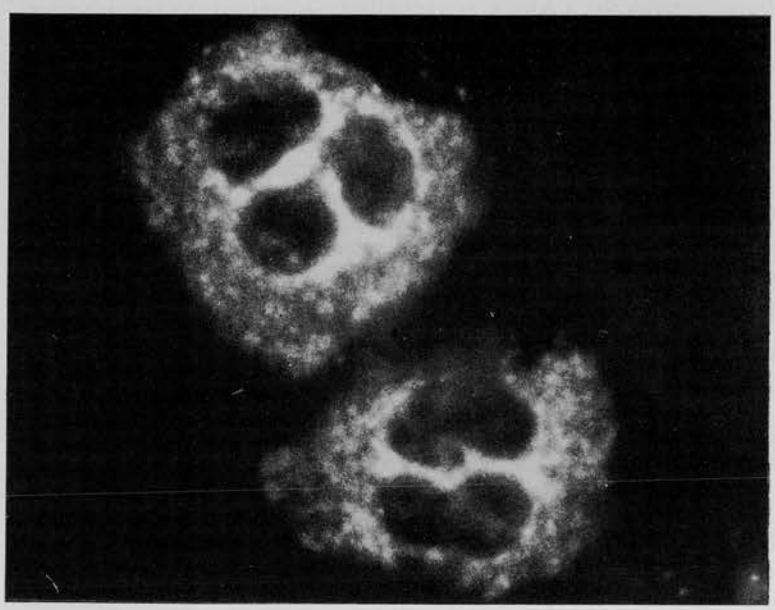

Fig 1 *Photomicrograph of cytospin preparation of normal neutrophils incubated sequentially with serum from a patient with Wegener's granulomatosis and fluoresceinated antihuman IgG, showing coarse granular cytoplasmic fluorescence.*

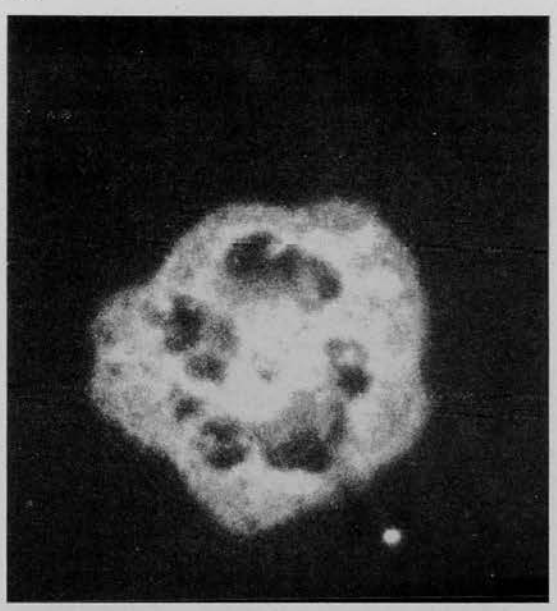

Fig 2 *Photomicrograph of a neutrophil cytospin preparation with a more diffuse cytoplasmic fluorescence than in figure 1 after incubation with serum from a patient with microscopic polyarteritis.*

unclear.[12] Primary vasculitis is generally accepted as having an immunological basis, probably with immune complexes damaging the vascular wall.[1] The diagnosis of Wegener's granulomatosis is frequently delayed because of the difficulty of obtaining definitive diagnostic material.[7 13] The discovery of ANCA (a generic rather than a specific title), which are thought to be specific for Wegener's granulomatosis, was therefore hoped to be an important advance in the recognition of this disease and its differentiation from other vasculitides, in particular microscopic polyarteritis.[2 4] The reported sensitivity of ANCA tests for active Wegener's granulomatosis has varied from 71% to 100%,[2 4 10] which is similar to the 78% in our series. We have found ANCA in a few cases of microscopic polyarteritis and Churg-Strauss syndrome but our specificity of 86% for Wegener's granulomatosis is high, and similar to the range of 88–100% reported in other series.[2 4 10 14] The precise figure for specificity is likely to vary a little as it will be influenced by the composition of the control group. These results are equivalent to accepted values for anti-double stranded DNA antibodies in systemic lupus erythematosus and antiacetylcholine receptor antibodies in myasthenia gravis.[4]

In addition to the very bright, granular cytoplasmic fluorescence that we regard as characteristic of Wegener's granulomatosis many samples had ANCA activity, albeit with less intense and diffuse fluorescence, including serum from four patients with Wegener's granulomatosis, six patients with microscopic polyarteritis, and two patients with Churg-Strauss syndrome. Other groups have reported diffuse fluorescence and have included this pattern under the title of ANCA.[2] It seems likely that more than one antigen is recognised by ANCA[15] and the pattern of fluorescence may relate to the antigen or antigens recognised by the antibodies present in the serum.[16] Many cases of connective tissue diseases as well as several cases of Paget's disease of bone were included in our study group. Diffuse, weak ANCA fluorescence has been described in serum from patients with primary biliary cirrhosis,[17] rheumatoid arthritis,[14] bronchogenic carcinoma,[10] and viral enteritis.[10] The meaning of these findings is not clear but they suggest that antibodies to neutrophil cytoplasmic antigens are a heterogeneous group of antibodies directed against different antigenic determinants and not a single entity. In cases of mixed connective tissue disease there is a well described antibody to extractable nuclear antigen.[18] Our present findings of cytoplasmic fluorescence in this condition may be the result of artefactual displacement of nuclear antigen during preparation of neutrophils for cytospin, in a way similar to the proposed displacement of nuclear c-myc oncoprotein during tissue fixation from the nucleus to the cytoplasm.[19] Patients with Wegener's granulomatosis do not usually have other specific autoantibodies.[4]

The weak ANCA fluorescence found in cases of Paget's disease of bone are of interest. Lockwood and colleagues[6] have proposed that the target antigen of ANCA in vasculitis is an epitope (antigenic determinant of known structure) derived from alkaline phosphatase, though this is controversial.[17 20 21] If alkaline phosphatase is an autoantigen, then ANCA may be a epiphenomenon related to increased serum concentrations of the enzyme as a result of neutrophil degranulation. In Paget's disease the serum concentration of bone alkaline phosphatase is raised, so possibly ANCA are the result of cross reactivity between epitopes of bone and neutrophil alkaline phosphatase. A similar argument may apply to similar antineutrophil cytoplasm fluorescence detected in cases of primary biliary cirrhosis.[17] In these cases the fluorescence is weak and difiuse, similar to that seen with rabbit anti-human alkaline phosphatase antiserum.[20]

In conclusion, the presence of ANCA giving bright granular cytoplasmic fluorescence is of considerable value in suggesting the diagnosis of Wegener's granulomatosis and, to a lesser extent, of some other vasculitides. The indirect immunofluorescence assay is rapid, reliable, reproducible, inexpensive, and within the capabilities of most laboratories. Further identification of the antigenic epitopes concerned may

increase both the specificity and the sensitivity of testing for ANCA in the diagnosis and follow up of Wegener's granulomatosis, and increase our understanding of the underlying disease process.

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References

1 Cupps TR, Fauci AS. *The vasculitides*. Philadelphia: Saunders, 1981:26.
2 Van der Woude FJ, Lobatto S, Permin H, *et al.* Autoantibodies against neutrophils and monocytes: tool for diagnosis and marker of disease activity in Wegener's granulomatosis. *Lancet* 1985;i:425–9.
3 Gross WL, Ludemann G, Kiefer G, Lehmann H. Anticytoplasmic antibodies in Wegener's granulomatosis. *Lancet* 1986;i:806.
4 Ludemann G, Gross WL. Autoantibodies against cytoplasmic structures of neutrophil granulocytes in Wegener's granulomatosis. *Clin Exp Immunol* 1987; **69**:350–7.
5 Rasmussen N, Wiik A, Hoier-Madsen M, Borregaard N, Van der Woude F. Antineutrophil cytoplasm antibodies. *Lancet* 1988;i:706–7.
6 Lockwood CM, Bakes D, Jones S, Whitaker KB, Moss DW, Savage COS. Association of alkaline phosphatase with an autoantigen recognised by circulating antineutrophil antibodies in systemic vasculitis. *Lancet* 1987;i:716–20.
7 Harrison DJ, Simpson R, Neary C, Wathen CG. Renal biopsy and antineutrophil antibodies and renal biopsy in the diagnosis and assessment of Wegener's granulomatosis. *Br J Dis Chest* 1988;**82**:398–404.
8 Lockwood CM. [Letter.] *Lancet* 1987;i:1489–90.
9 Wathen CG, Harrison DJ. Circulating antineutrophil antibodies in systemic vasculitis. *Lancet* 1987;i:1037.
10 Venning MC, Arfeen S, Bird AG. Antibodies to neutrophil cytoplasmic antigen in systemic vasculitis. *Lancet* 1987;ii:850.
11 Wathen CG, Harrison DJ, Bell D, Davies J, Douglas AC. Determination of disease activity of Wegener's granuloma. *Scott Med J* 1988;**33**:221.
12 McCluskey RT, Fienberg R. Vasculitis in primary vasculitides, granulomatoses, and connective tissue disease. *Hum Pathol* 1983;**14**:305–15.
13 Leavitt RY, Fauci AS. Pulmonary vasculitis. *Am Rev Respir Dis* 1986;**134**:149–66.
14 Savage COS, Jones S, Winearls CG, Marshall PD, Lockwood CM. Prospective study of radioimmunoassay for antibodies against neutrophil cytoplasm in the diagnosis of systemic vasculitis. *Lancet* 1987;i: 1389–93.
15 Harrison DJ, Kharbanda R. The characterisation of antibodies to neutrophil cytoplasmic antigens in systemic vasculitis. *Nephrol Dial Transplant* 1988;**3**:526.
16 Penning CA, Jones S, Lockwood CM. Antibody binding profiles of sera from systemic vasculitis patients. *Nephrol Dial Transplant* 1988;**3**:527.
17 Gross WL, Ludemann J, Schroder J-M. Antineutrophil cytoplasm antibodies in Wegener's granulomatosis are not directed against alkaline phosphatase. *Lancet* 1987;i:1488–9.
18 Sharpe GC. Mixed connective tissue disease—an apparently distinct rheumatic disease syndrome associated with a specific antibody to an extractable nuclear antigen (ENA). *Am J Med* 1972;**52**:148–59.
19 Williams ARW, Wyllie AH, Piris J. Immunohistochemical demonstration of p62 myc oncogene product in human colonic neoplasms [abstract]. *J Pathol* 1987;**152**:234A.
20 Rasmussen N, Borregaard N, Wiik A. Antineutrophil cytoplasm antibodies in Wegener's granulomatosis are not directed against alkaline phosphatase. *Lancet* 1987;i:1488.
21 Goldschmeding R, Tetteroo PAT, Von der Borne AEG, Kallenberg CGM. Antineutrophil cytoplasm antibodies in Wegener's granulomatosis are not directed against alkaline phosphatase. *Lancet* 1987;i:1489.